Contrasting responses of bacterial and fungal communities to plant litter diversity in a Mediterranean oak forest

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Abstract

Tree species diversity of forested ecosystems control the diversity of leaf litter inputs to the soil, with cascading effects on the microbial communities colonizing decomposing litter. However, the extent to which bacterial and fungal communities inhabiting the litter layer are affected by shifts in tree species diversity is not well understood. To investigate the role of litter species diversity, litter species identity and litter functional traits on bacterial and fungal communities of a typical Mediterranean oak forest, we set up a yearly field litterbag experiment that considered leaf litter mixtures of four abundant species: *Quercus pubescens*, *Acer monspessulanum*, *Cotinus coggygria* and *Pinus halepensis*. We found that both bacterial and fungal communities varied strongly during decomposition but showed distinct succession patterns. Both communities were also strongly influenced by litter species diversity, litter identity and litter functional traits. The intensity and the direction of these effects varied during decomposition. Litter diversity effects were mediated by litter species composition rather than litter species richness, highlighting the importance of litter species identity - and associated litter traits - as drivers of microbial communities. Both the "mass-ratio hypothesis", measured through the community weighted mean (CWM) litter traits, and the "niche complementarity hypothesis", measured through the functional dissimilarity (FD) of litter traits, contributed to litter diversity effects, with a greater relative importance of FD compared to CWM, and with an overall stronger impact on fungal than on bacterial communities. Interestingly, increasing FD was related to decreasing bacterial diversity, but increasing fungal diversity. Our findings provide clear evidence that any alteration of plant species diversity produces strong cascading effects on microbial communities inhabiting the litter layer in the studied Mediterranean oak forest.

Keywords: Bacteria; fungi; litter decomposition; litter traits; Mediterranean forest; plant-soil interaction

1. Introduction

Alterations of plant communities due to ongoing global change, including climate, land-use and resource availability changes, have already been observed and are presumed to continue (Vitousek et al., 1997; Walther et al., 2002; van der Knaap et al., 2018). Studies of the relationship between plant species diversity and ecosystem functioning have received growing attention in the last decade (Diaz and Cabido, 2001; Loreau et al., 2001). The majority of such studies investigated how plant diversity affects litter decomposition efficiency (Gartner and Cardon, 2004; Hättenschwiler et al., 2005), but only a few have examined how it affects the organisms running the process (Bardgett and Shine, 1999; Leloup et al., 2018). Soil microorganisms are indeed major drivers of litter decomposition and mineralization (Bardgett, 2005; de Graff et al., 2010) and their responses to changes of leaf litter diversity should interact with the decomposition process efficiency.

Litter microbial communities are strongly influenced by the quantity and the quality of litter input from the plant community (Calderon et al., 2001; Lalor et al., 2007). Differences in litter quality among tree species have been reported to affect the abundance and composition of soil bacterial and fungal communities (Grayston et al., 1998; Aponte et al., 2013; Prescott and Grayston, 2013). Both nitrogen (N) and phosphorus (P) availabilities actually shape microbial decomposer communities as they are often limiting elements in the soil and play a central role in resource competition (Cleveland and Liptzin, 2007; Mooshammer et al., 2014). Other plant litter traits such as secondary metabolites are recognized to repress the biomass and activity of microbial communities (Fierer et al., 2001; Ushio et al., 2013; Chomel et al., 2014, 2016). For instance, Chomel et al. (2014) observed a negative correlation between phenolic concentrations and fungal biomass during litter decomposition in a Mediterranean pine forest, calling attention

74 to the inhibitory effect of phenolic compounds from pine leaf litter toward fungi (Kainulainen
75 et al., 2003; Kraus et al., 2003; Hättenschwiler et al., 2005). Likewise, Amaral and Knowles
76 (1998) reported that monoterpenes inhibited the activity and growth of certain soil microbial
77 groups while stimulating others.

78 However, in most natural ecosystems, litter material from various plant species
79 decomposes together. As key actors of litter decomposition, microbial communities are likely
80 responding to the variation in species composition of the litter input that determines litter
81 functional diversity. According to the "mass-ratio hypothesis" (Grime, 1998), ecosystem
82 properties are strongly related to the relative input of each species, implying that functional
83 traits of the dominant plant species mainly determine ecosystem function (Garnier et al., 2004)
84 and thus the litter trait control over microbial communities changes along community-weighted
85 mean (CWM) trait values. Alternatively, the "niche complementarity hypothesis" argues that
86 the functional diversity (FD) of trait-values promotes an ecosystem's functioning (Petchey and
87 Gaston, 2006; Diaz et al., 2007), for instance, by improving the availability of complementary
88 resources for microbial communities (Wardle et al., 1997; Barantal et al., 2014; Handa et al.,
89 2014). These two mechanisms can operate simultaneously by affecting microbial communities
90 as a result of plant (litter) composition change. However, our knowledge about the relative
91 importance of these theories in controlling microbial decomposer communities is very limited.

92 The influence of tree species diversity on litter microbial communities has been studied
93 in boreal, temperate and tropical ecosystems, while information from Mediterranean
94 ecosystems is extremely scarce (e.g. Shihan et al., 2017; Santonja et al., 2017a, 2017b).
95 Mediterranean forests have a different species composition than temperate forests due to the
96 Mediterranean climate characteristics that have shaped species distribution (Quézel and Médail,
97 2003). Remarkable contrasts of temperature and humidity across seasons, and in particularly
98 the summer drought period, correspond to particular leaf litter traits, including secondary

99 metabolites (Chomel et al., 2014; Chomel et al., 2016; Hashoum et al., 2017), that have the
100 potential to influence microbial decomposer communities (Schimel et al., 2007; Williams and
101 Rice, 2007; Brockett et al., 2012).

102 In this study we assessed the potential consequences of a forest composition shift on
103 microbial communities associated with decomposing leaves, including both loss and gain of
104 tree species. We conducted a full-factorial *in situ* decomposition experiment over a gradient of
105 litter species diversity in a Mediterranean downy oak forest. Downy oak (*Quercus pubescens*
106 Willd.) is broadly distributed from northern Spain to the Caucasus (Quézel and Médail, 2003),
107 and is the dominant species structuring many forests of the northern parts of the Mediterranean
108 basin. We used leaf litter from the three dominant woody plant species naturally present in the
109 forest (*Q. pubescens*, *Acer monspessulanum* L. and *Cotinus coggygria* Scop.) and one pine
110 species (*Pinus halepensis* Mill.), which may become more frequent in downy oak forest in the
111 future in response to climate change (i.e. under warmer and drier environment; Gaucherel et
112 al., 2008; Bede-Fazekas et al., 2014). We aimed to determine the effects of (i) litter species
113 richness, (ii) litter species composition, (iii) litter species identity and (iv) litter functional trait
114 values on the dynamics of microbial communities, during one-year leaf litter decomposition.
115 Using fingerprinting methods, we explored in parallel fungal and bacterial community
116 dynamics at six time points, expecting to reveal differences across microbial decomposers
117 groups that have distinct trophic niches (Boer et al., 2005; Buée et al., 2009; Lopez-Mondejar
118 et al., 2015). We hypothesized that H1: microbial diversity and community structure associated
119 to decomposed leaves or needles change with decomposition time, as the litter quality changes
120 over the course of decomposition (Snajdr et al., 2011; Baldrian et al., 2012); H2: microbial
121 diversity increases with the increase of litter species richness, by increasing resource diversity
122 and microhabitat heterogeneity; H3: microbial community parameters (diversity and
123 community structure) are also determined by the plant identity in the litter mixture, as

differences of litter quality among plant species would lead to distinct effects on microbial community parameters; H4: microbial diversity responds to "niche complementarity hypothesis" rather than to "mass-ratio hypothesis", as litter mixtures with contrasting litter quality improve the availability of different resources and microhabitat for microorganisms. We also hypothesized that H5: the relationships between microbial decomposers and litter diversity decrease with decomposition time, as resource diversity changes during leaf litter decomposition, leading to homogenization of litter quality across litter mixtures.

2. Materials and methods

2.1. Study site

This study was conducted in the Oak Observatory at the OHP (O_3HP) experimental site located in the research center "Observatoire de Haute Provence", 60 km north of Marseille, South of France (43°56'115" N, 05°42'642" E). The site is 680 m above sea level, and presents a mean annual temperature of 11.9 °C and a mean annual precipitation of 830 mm (1967 - 2000 period, WMO standard temperature and precipitation: 1960-2003 St Michel l'Observatoire / Meteo France 04192001; 2003-2010 Dauphine / Meteo France 04068001 - on and close to the OHP, respectively). Temperature and rainfall during the experiment showed a seasonal distribution characteristic of a Mediterranean climate, with maximum rainfall in June and October, maximum temperature in July and August, and a dry summer season lasting less than two months, typical of a supramediterranean bioclimatic stage (Supplementary Fig. S1). According to the French Référentiel Pédologique (Baize and Girard, 1998), the soil is a pierric calcosol (with S horizon between limestone rocks) or a calcarisol when limestone appears less than 25 cm deep. The pH is between 6.5 and 7.5 for the A horizon and 7.5 the for S horizon.

148 According to the WRB classification (IUSS Working group WRB, 2006) the soil can be
149 classified as a mollic leptosol.
150 The study site is a Mediterranean natural old-growth oak forest belonging to the site
151 Natura 2000 "FR9302008 Vachères", in which 53 different plant species were identified. This
152 forest already existed in the late 18th century (Hilaire et al., 2012) and was managed for
153 centuries by coppicing. *Q. pubescens* (Downy oak: 75% coverage) and *A. monspessulanum*
154 (Montpellier maple: 25% coverage) are the two dominant tree species, with understory
155 vegetation dominated by *C. coggygria* (smoke tree: 30% coverage). *P. halepensis* (Aleppo pine)
156 is present very close to the downy oak forest but only in open environments.
157
158 ***2.2. Experimental set-up***
159 This experiment used litter of four species: *A. monspessulanum*, *C. coggygria*, *P.*
160 *halepensis* and *Q. pubescens* that will be named *Acer*, *Cotinus*, *Pinus* and *Quercus* hereafter.
161 Freshly abscised leaves of the four species were collected over the whole period of maximum
162 litter fall from October to November using litter traps suspended under the relevant species.
163 Leaves were dried at ambient temperature and stored until the beginning of the experiment.
164 Leaf litter decomposition was studied for 320 days using the litterbag method (Swift et
165 al., 1979). Litterbags measuring 20 cm × 20 cm with a mesh size of 4 mm were filled with 10
166 g of dry leaf litter and placed in the field in January. Fifteen species mixtures were made with
167 an equal partitioning on a dry mass basis between species: a single-mixture litter for each
168 species (four types), all possible two-species mixture combinations (six types), all possible
169 three-species mixtures (four types) and a four-species mixture. There were thus 15 treatments,
170 comprising four levels of species richness: 1, 2, 3 and 4 species. A total of 360 litterbags (15
171 types × 6 sampling dates × 4 replicates) were used for the experiment. We used a nested
172 experimental design with four blocks of 100 m^2 (spaced of 10 m) where replicates were

173 randomly scattered and spaced of 1 m in each block. We retrieved four replicates of each
174 treatment (i.e. one per block) every 50 (all the sampling dates) or 70 days (the last sampling
175 date, because violent rain events prevented sampling).

176 After removal, litterbags were immediately sealed in plastic bags to prevent loss of litter
177 material, and transported to the laboratory. Leaves were separated according to species, which
178 was possible even with small fragments of litter owing to marked morphological differences
179 among species. To prevent contamination of litter by soil, we wiped each leaf thoroughly before
180 analysis. Samples were freeze-dried (Lyovac GT2®), weighed and ground to powder.

181

182 ***2.3. Litter traits***

183 Four litter samples of each species were taken to determine the initial characteristics of
184 the litter (Supplementary Table S1). Carbon (C) and nitrogen (N) content of the litters were
185 determined by thermal combustion on a Flash EA 1112 series C/N elemental analyzer (Thermo
186 Scientific, USA).

187 Phosphorus (P) was extracted with 20 ml of nitric acid from remaining dry ash after
188 combustion of 0.5 g of subsamples at 500 °C for 5 h in a muffle furnace. The pH was adjusted
189 to 8.5 with a 40% NaOH solution; 1 ml of each sample, 0.2 ml of mixed reagent (emetic tartar
190 and ammonium molybdate solution), 0.04 ml of ascorbic acid and 0.76 ml of distilled water
191 were placed directly in a spectrophotometer microcuvette. After 150 min, the reaction was
192 completed, and phosphorus concentration was measured at 780 nm with a UV/Vis
193 spectrophotometer (Thermo Scientific, USA).

194 Total phenolic concentrations were measured colorimetrically by the method of
195 Peñuelas et al. (1996) using gallic acid as a standard. A 0.25 g litter sample was dissolved in 20
196 ml of a 70% aqueous methanol solution, shaken for 1 h and then filtered (0.45 µm filter); 0.25
197 ml of filtered extract was mixed with 0.25 ml Folin-Ciocalteu reagent (Folin and Denis, 1915),

198 0.5 ml of saturated aqueous Na_2CO_3 (to stabilize the color reaction) and 4 ml of distilled water.
199 After 60 min, the reaction was completed, and concentration of phenolics was measured at 765
200 nm on a UV/Vis spectrophotometer (Thermo Scientific, U.S.A.).

201 Terpenes were extracted from 0.5 g of litter sample with 5 ml of dichloromethane, and
202 50 µl of an internal standard (dodecane) was added. The samples were filtered and then
203 analyzed by gas-chromatography/mass-spectrometry (GC-MS) on an Agilent HP6890 system
204 equipped with an MSD5973 Network mass detector, an ALS7673 automatic injector and an
205 HP5-MS apolar column (30 m × 0.25 mm × 0.25 µm; J&W Agilent Technologies, Crawford
206 Scientific, Lanarkshire, Scotland, UK). Retention indices were determined relative to injected
207 Wisconsin Diesel Range Hydrocarbons (Interchim, Montluçon, France) and confirmed by
208 comparison against expected literature values (Adams, 2007). Finally, the total terpene
209 concentration was measured as the sum of each of the individual terpene compounds.

210 To assess the "mass-ratio hypothesis", we calculated the community-weighted mean
211 traits (CWM) of the litter mixtures as the average trait values of the litter mixtures following
212 Garnier et al. (2004) as $Trait_{CWM} = \sum_{i=1}^{n} p_i \times trait_i$ where p_i is the relative abundance for
213 species i and $trait_i$ is the trait value for species i. To assess the "niche complementarity
214 hypothesis", we calculated functional dissimilarity (FD) of litter mixtures according to Rao's
215 quadratic entropy (Botta Dukat, 2005) for each litter mixture as: $\text{Trait}_{FD} = \sum_{i=1}^{n} \sum_{j=1}^{n} pipj * dij$
216 where p_i and p_j are the relative abundance for shrub species i and j in the litter mixture, and d_{ij}
217 the Euclidian distance between species i and j for the trait considered. Because the measured
218 traits differ in their units, we used normalized values (using a z-scored standardization so as to
219 get a mean of zero and a standard deviation of one) to calculate functional dissimilarity.
220 According to the "mass-ratio hypothesis", the highest scores of CWM are reached for species
221 within the mixture that exhibit the highest trait values. On the other hand, according to the
222 "niche complementarity hypothesis", the highest scores of FD are reached for very dissimilar

223 litter species within the mixture. Since we predicted that microbial diversity responds to the
224 "niche complementarity hypothesis" rather than to the "mass-ratio hypothesis" (i.e. research
225 hypothesis H4), we expect that the increase in microbial diversity to be better correlated to the
226 increase in FD values than to the increase in CWM values.
227
228 ***2.4. Bacterial and fungal communities***
229 Extraction of microbial cells from litter was achieved by washing 100 mg of freeze-
230 dried ground litter material with 1.5 ml of sterile saline solution (0.9 % NaCl; w/v). The
231 suspension was mixed by vortexing for 5 s and then centrifuged for a few seconds in order to
232 pellet the leaf residues. Microorganisms were recovered from the supernatant following transfer
233 to a new microtube and being centrifuged (16000 g, 30 min) to form a microbial pellet that was
234 frozen at -20°C until DNA extraction. Total DNA was extracted from each microbial sample
235 by using the PowerSoil-htp 96 wells DNA isolation kit (MoBio, Laboratories, Inc.), adjusting
236 the protocol to the use of a vacuum manifold. DNA samples were stored at -20°C until analysis.
237 We used Automated Ribosomal Intergenic Spacer Analysis (ARISA) to characterize
238 bacterial communities (Ranjard et al., 2001), and terminal Restriction Fragment Length
239 Polymorphism (tRFLP) to characterize fungal communities (Liu et al., 1997). The bacterial
240 16S-23S rDNA Intergenic Spacer (ITS) was amplified using the primers [6fam]-s-d-Bact-1522-
241 b-S-20 (5'-TGCGGCTGGATCCCCTCCTT-3') and L-D-Bact-132-a-A-18 (5'-
242 CCGGGTTTCCCCATTCGG-3'). The fungal 18S-5,8S ITS1 region was amplified using the
243 primers [HEX]-ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3'; Gardes and Bruns, 1993)
244 and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White et al., 1990). Both PCR reactions
245 were performed in 20 µl mixtures containing 200 µM dNTPs, 5 % (v:v) DMSO, 0.5 µM of each
246 primer and 0.25 units GoTaq DNA-polymerase with the corresponding 5X PCR buffer
247 (Promega). After initial denaturation at 94°C for 5 minutes, we ran 35 cycles of: denaturation

248 at 94°C for 1 minute, followed by annealing at 55°C for 1 minute, elongation at 72°C for 1
249 minute and a final elongation at 72°C for 10 minutes. PCR products of the fungal ITS were
250 digested by adding 5 U of *Hinf*I restriction enzyme and 2 µl of the corresponding restriction
251 buffer (Fermentas). Following verification of the ARISA and tRFLP products by using 2%
252 NuSieve-agarose gel electrophoresis, these products were diluted with sterile distilled water
253 (1/20), and analyzed with a capillary sequencer ABI 3730 (Applied Biosystem). One microliter
254 of the diluted sample was mixed with 0.8 µl of GeneScan-2500 ROX-labelled size standard and
255 8 µl of deionized formamide; denaturation was completed at 95°C for 5 min before capillary
256 electrophoresis in the POP-7 polymer, during 3 h with 7.5 kV run-voltage. The output series of
257 peak-sizes corresponding to bacterial or fungal operating taxonomic units (OTUs) were
258 analyzed by using the GeneMapper® v4.1 program (Applied Biosystem). Size standard peaks
259 were defined individually, parameters of the internal AFLP-method were set up in order to
260 detect peak-sizes in the 300-1200 bp range, with bin-windows set up to one bp and lower
261 detection limits of peak-heights fixed individually at values ranging from 1 to 100 Raw
262 Fluorescent Units (RFUs).
263
264 ***2.5. Statistical analyses***
265 Statistical analyses were performed with the PRIMER-E software (version 6.1, Primer-
266 E Ltd, Plymouth, United Kingdom) for multivariate analyses, and with the R software (version
267 3.3.1, The R Foundation for Statistical Computing, Vienna, Austria) for univariate analyses.
268 Bacterial and fungal OTU (Operational Taxonomic Unit) - abundance matrices based
269 on ARISA and tRFLP fingerprints were analyzed with PRIMER-E. The "DIVERSE" routine
270 was ran to obtain the Shannon's indices of diversity ($H' = -\Sigma\ p_i \times \log_{10} p_i$), where p_i is the
271 proportion of the total abundance arising from the i^{th} species. The abundance value of each
272 OTU was standardized by total abundance of the sample and then log-transformed (Log $X+1$)

before generating Bray-Curtis similarity matrices with minimum transformed-value as a dummy variable. To visualize distance between samples, we used Principal Coordinate Ordination (PCO) plots. We assessed whether microbial community structure differed according to the time of decomposition, the litter diversity and the presence of plant species in the litter mixtures by using analysis of similarity (ANOSIM) routine and multivariate analysis of variance (PERMANOVA) that was set up with 9999 permutations and unrestricted permutation of raw data method. The dispersion of the microbial communities shown by the PCO was studied using the PERMDISP routine. We determined bacterial and fungal communities' turnover rates during litter decomposition (Supplementary Table S2; Supplementary Fig. S2) for each litter mixture treatment, as the linear regression estimate of Bray-Curtis dissimilarity changes, between samples collected after 50 days decomposition and all successive collection dates (i.e. following 100, 150, 200, 250 and 320 days decomposition).

We used a general linear model approach to test for the effects of litter diversity (separated in i) litter species composition, ii) litter species richness, iii) litter species identity and iv) litter functional traits) on bacterial and fungal diversity. Due to the large number of potentially important predictors, we carried out three distinct statistical models. The first model tested the impact of litter diversity (decomposed in litter species richness and litter species composition), time of decomposition (expressed as harvest date) and block on bacterial and fungal diversity (expressed as the Shannon-Wiener index). The second model tested the impact of litter species identity (i.e. the presence/ absence of a particular species), time of decomposition and block on bacterial and fungal diversity.

For a more detailed understanding of how the diversity of leaf litter affected microbial diversity, we evaluated the effects of mean traits ($Trait_{CWM}$) and functional trait dissimilarities ($Trait_{FD}$) of the 8 measured litter traits (Supplementary Table S1). A principal component analyses (PCA), based on these 8 litter traits across litter mixtures, allowed to define the two

first components of each PCA (i.e. CWM1 and CWM2, and FD1 and FD2) as characteristic vectors of CWM and FD, respectively. We then performed a third model in order to decipher the relative contributions of CWM1, CWM2, FD1, FD2, time of decomposition and block on bacterial and fungal diversity.

Finally, we performed regressions analyses to test for the relationships between litter functional traits ($Trait_{CWM}$ and $Trait_{FD}$) and bacterial and fungal community turnover rates (Supplementary Table S2).

3. Results

3.1. Microbial community dynamics during decomposition

Overall, 746 bacterial and 448 fungal OTUs were detected in the litter during this experiment. Time of litter decomposition explained the largest proportion of the variation in microbial diversity (Tables 1-3). Bacterial diversity increased at the beginning of litter decomposition and remained constant from 150 days to the end of the experiment (Fig. 1a). Fungal diversity increased during decomposition and reached its maximum value after 320 days of litter decomposition (Fig. 1d). Microbial community dissimilarities varied importantly during time of litter decomposition (PERMANOVA, bacterial community: Pseudo-F = 15.5, P < 0.001; fungal community: Pseudo-F = 19.1, P < 0.001). Both bacterial and fungal communities changed during the decomposition experiment (Supplementary Fig. S3). While the dissimilarity among fungal communities remained steady during decomposition (Supplementary Fig. S3b), the dissimilarity among bacterial communities decreased leading to the homogenization of bacterial community across mixtures over time (Supplementary Fig. S3a).

3.2. Litter diversity effects on microbial communities

Litter species composition accounted for a higher portion of the overall variance of microbial diversity than litter species richness (Table 1; Supplementary Table S3). Bacterial diversity was higher in the three- and four-species litter mixtures compared to the single and two-species litter mixtures (Fig. 1b). Fungal diversity was the lowest in single-species litter mixtures; it increased with increasing species richness and reached its maximum value in the three- and four-species litter mixtures (Fig. 1e). Microbial diversity differed among the 15 litter species combinations (Figs. 1c and 1f). However, the litter mixture effects on microbial diversity, including both litter species composition and litter species richness, varied during the course of the decomposition (Litter species richness × Time and Litter species composition × Time interactions, Table 1). Specifically, bacterial diversity was partially affected by litter species richness (i.e. after 100, 200 and 320 days of decomposition; Supplementary Table S3; Figs. 2b, 2d and 2f), while fungal diversity was affected by litter species richness during all the decomposition process, but not after 320 days of decomposition (Supplementary Table S3; Fig. 2l). Litter species composition affected microbial diversity throughout the decomposition periods, except for bacterial diversity after 150 days (Supplementary Table S3).

Litter species richness influenced the fungal community dissimilarity by increasing the resemblance of community in multi-species litter mixtures compared to single-species litter (PERMANOVA, Pseudo-F = 2.67, P < 0.01). On the contrary, litter species richness had no effect on the bacterial community dissimilarity (PERMANOVA, Pseudo-F = 1.08, P = 0.2403). Litter species composition strongly influenced fungal communities, but had reduced effects on bacterial communities (Supplementary Fig. S4b).

3.3. Litter identity effects on microbial communities

Measuring the response of microbial diversity to the presence of each plant species in the mixtures revealed the major effects of *Quercus*, that increased both bacterial and fungal diversities all along the decomposition process and the effect of *Pinus* that increased specifically fungal diversity at most sampling time. Except for *Quercus*, the effects of the three other plant species on microbial diversity strongly depended on decomposition time (Table 2). The presence of *Acer* enhanced bacterial diversity after 100 and 320 days of decomposition (Time × *Acer* presence interaction, Table 2; Fig. 3a). *Cotinus* decreased both bacterial and fungal diversities after 50 days of decomposition and, on the opposite, positively affected both bacterial and fungal diversities at later decomposition stages (Time × *Cotinus* presence interaction, Table 2; Figs. 3b and 3f). The presence of *Pinus* enhanced fungal diversity until 250 days of decomposition, whereas *Pinus* decreased both bacterial and fungal diversities after 320 days of decomposition (Time × *Pinus* presence interaction, Table 2; Figs. 3c and 3g). Presence of *Quercus* enhanced both bacterial and fungal diversities throughout the decomposition process (Table 2; Figs. 3d and 3h).

Microbial community dissimilarities also differed according to the identity of the plant species in the litter mixture (Supplementary Fig. S5). The bacterial community dissimilarity was affected by the presence of *Acer* (ANOSIM, $R = 0.16$, $P < 0.001$; Supplementary Fig. S5a) and *Pinus* (ANOSIM, $R = 0.13$, $P < 0.001$; Supplementary Fig. S5e), but not by the presence of *Cotinus* or *Quercus*. The fungal community dissimilarity was mainly affected by the presence of *Pinus* (ANOSIM, $R = 0.32$, $P < 0.001$; Supplementary Fig. S5f) and to a lower extent by *Acer* (ANOSIM, $R = 0.18$, $P < 0.001$; Supplementary Fig. S5b). Interestingly, the influence of *Quercus* on fungal community dissimilarity was covered by the influence of *Pinus* (ANOSIM, $R = 0.16$, $P < 0.001$; Supplementary Fig. S5h). In other words, the presence of *Quercus* had a different effect on litter mixtures either containing or excluding *Pinus*.

3.4. Control of microbial communities by mass ratio or niche complementarity hypothesis

Principal component analysis of CWM traits showed that the first PCA axis (CWM1), explaining 66.3% of traits variation, was determined mainly by high C and terpen concentrations and low phenolic concentration (Fig. 4a). The second PCA axis (CWM2), explaining 26.4% variation, was related mainly to high values of C:N and C:P ratios, and low values of N and P concentrations (Fig. 4a). Principal component analysis of FD traits showed that the first PCA axis (FD1), explaining 45% of trait dissimilarity variation, was related mainly to increasing dissimilarities of phenolic concentration, and of C:P and N:P ratios (Fig. 4b). The second axis (FD2), explaining 30.5% variation, was related mainly to increasing dissimilarity of terpen and C concentrations, and of C:N ratio (Fig. 4b).

When evaluating jointly the effects of CWM and FD traits, we found that bacterial diversity was mostly controlled by time of decomposition, FD2 and the interaction FD2 × time of decomposition (Table 3). Increasing FD2 scores (i.e. decreasing functional dissimilarity of terpen, C and C:N ratios) were related to higher bacterial diversity index after 200, 250, and 320 days of decomposition, as indicated by the FD2 × time of decomposition interaction (Table 3; Fig. 5) and by the negative relationship between the bacterial communities turnover rate and FD2 (Table 4).

In contrast to bacterial diversity, both CWM and FD traits strongly controlled fungal diversity (Table 3). Fungal diversity was significantly affected by time of decomposition, CWM1, FD2 as well as the interactions CWM1 × time of decomposition, CWM2 × time of decomposition and FD2 × time of decomposition (Table 3). Increasing CWM1 scores (correlated mainly to decreasing phenolic concentration) were related to higher fungal diversity, except after 320 days of decomposition (CWM1 × time of decomposition interaction, Table 3). Increasing CWM2 scores (mainly correlated to increasing values of C:N and C:P ratios) were related to higher fungal diversity after 50 and 100 days of decomposition (CWM2 × time of

decomposition interaction, Table 3). The relationship between FD2 and fungal diversity opposed to that observed with bacterial diversity: increasing FD2 scores (i.e. correlated mainly to decreasing C, terpen and C:N dissimilarities) were related to lower fungal diversity, except after 320 days of decomposition (FD2 × time of decomposition interaction, Table 3; Fig. 5).

The relationships between the dynamic of fungal diversity during litter decomposition and both CWM1 and FD2 values were confirmed by similar relationships with fungal communities' turnover rate (i.e. negative correlation of fCTR with CWM1 and positive correlation of fCTR with FD2; Table 4).

4. Discussion

In line with our first hypothesis, microbial community's changes were important across decomposition time. While the fingerprinting methods used are likely to detect DNA of living microorganisms and relic DNA that is not fully degraded during the experiment (Carini et al., 2017), our result is consistent with previous studies on the seasonal variation of microbial communities, reporting that variations in soil moisture, temperature or leaf litter chemistry during decomposition were most likely causes of changes in microbial communities (Snajdr et al., 2011; Voriskova et al., 2014; Lopez-Mondejar et al., 2015; Purahong et al., 2015). The bacterial community showed a faster dynamic and reached a higher diversity than the fungal community, which confirms previous studies (Baldrian et al., 2012; Urbanova et al., 2015; Santonja et al., 2017a). Interestingly, we observed that the community heterogeneity among litter mixtures remained constant along the decomposition process for fungi, while it gradually disappeared for bacteria (Supplementary Fig. S3). This finding supports the idea that the rapid establishment of bacterial diversity is driven by the availability of simple compounds at early decomposition stages (Moorhead and Sinsabaugh, 2006); then, simple compounds decrease in quantity to the advantage of recalcitrant compounds, which triggers selective changes in the

bacterial decomposer community. Comparatively, the fungal decomposer community showed an apparently lower diversity that gradually established to its maximum value at later decomposition stages. These results suggest that diversification of the fungal decomposer community at advanced litter decomposition stages relies on competition for recalcitrant compounds utilization and to late colonization of the remaining leaf tissue (Moorhead and Sinsabaugh, 2006).

In agreement with our second hypothesis, microbial diversity increased with litter species richness. Previous studies have provided evidence that microbial biomass and diversity respond positively to litter mixing (Blair et al., 1990; Bardgett and Shine, 1999; Chapman and Newman, 2010; Santonja et al., 2017a), with differences in litter properties causing resource diversification and habitat heterogeneity for microbial decomposers (Tilman et al., 1997; Hooper and Vitousek, 1998; Hättenschwiler et al., 2011). Unlike Santonja et al. (2017a) who reported that only fungal diversity increased after one year of litter decomposition in a Mediterranean oak shrubland, here we observed that both bacterial and fungal diversities increased during the process in the studied Mediterranean oak forest. We also clearly demonstrated that the litter diversity effects are mediated by litter species composition rather than litter species richness, highlighting the importance of litter species identity in litter mixtures as a driver of microbial community diversity. We partially confirmed our hypothesis that microbial responses to litter species diversity (both composition and richness) decrease with decomposition time. Because it is generally assumed that initial differences in litter quality among different plant species converge to similar quality during litter decomposition (e.g. Moore et al., 2005; Preston et al., 2009), we expected to observe greater litter diversity control of microbial communities during the early stages of decomposition. Interestingly, litter diversity effects varied during the experiment but were not restricted to the early decomposition stages.

In line with our third hypothesis, microbial community proxies were sensitive to the litter species identity, as the presence of the four plant species affected - positively or negatively - microbial diversities. Previous studies also highlighted the importance of tree species identity as drivers of microbial communities, rather than changes in tree species richness (Scheibe et al., 2015; Urbanova et al., 2015). As for the litter diversity effect, the litter identity effect on microbial communities was irregular across decomposition times. Interestingly, the positive effects of *Q. pubescens* on both bacterial and fungal diversities remained constant during all the decomposition process. Since *Q. pubescens* is the dominant tree in the studied Mediterranean oak forest, microorganisms could be well adapted to colonize and degrade this litter type. *A. monspessulanum* enhanced sporadic bacterial diversity, but showed a noticeable effect on microbial community dissimilarities, suggesting an important contribution on bacterial community composition. This effect is probably related to high contents of N and P (Supplementary Table S1) that are generally limiting nutrients in the soil and would be mostly favorable to heterotrophic bacterial decomposers (Cleveland and Liptzin 2007; Mooshammer et al., 2014). *C. coggygria* expressed negative effects on both bacterial and fungal diversities only at the earliest decomposition stage, which then reverted to a positive effect at later decomposition stages. This could be attributed to its strong initial phenolic content (Supplementary Table S1) that was shown to affect microbial colonization of leaf litter (Chomel et al., 2014) and is later on rapidly lixiviated (73% phenolic content loss after 100 days of decomposition; Santonja et al., 2015). As the alien species of the study, *P. halepensis* is the only coniferous species among the four studied woody-plant and is characterized by the highest terpene content. Its presence in the litter mixtures noticeably enhanced fungal diversity until later decomposition stages (although this effect decreased during the experiment; Fig. 3g) and also showed striking effects on fungal community dissimilarities. These results suggest that terpenes favor fungal decomposer diversity. Interestingly the effect of *Q. pubescens*

472 superimposed to the effects of *P. halepensis* leading to four distinct fungal communities
473 (Supplementary Fig. S5h). Overall, the fungal community composition showed essentially
474 more susceptibility to the identity of plant species present in the leaf litter mixtures than the
475 bacterial community composition (Supplementary Fig. S4), stressing the tight and specialist
476 relationship between plant species and fungal decomposer community, compared to the more
477 relaxed and generalist relationship with bacterial decomposer community (Moorhead and
478 Sinsabaugh, 2006).

479 In line with our fourth hypothesis, both mass-ratio (measured through community
480 weighted mean (CWM) of litter traits) and niche complementarity (measured through
481 functional dissimilarity (FD) of litter traits) hypotheses contributed to the litter diversity effects
482 on microbial decomposer communities, with a greater relative importance of the niche
483 complementary compared to the mass ratio hypothesis. According to the niche complementarity
484 hypothesis, litter mixtures with contrasting litter quality improve the availability of different
485 resources for decomposers (Tilman et al., 1997; Hooper and Vitousek, 1998; Schimel and
486 Hättenschwiler, 2007). But, surprisingly, increasing functional dissimilarity of litter traits -
487 hence resource availability, was unfavorable to the bacterial community turnover rate and
488 diversity while conversely, it was beneficial to the fungal community turnover rate and diversity
489 in the studied Mediterranean oak forest. In contrast to bacterial diversity, the first and second
490 components of the CWM PCA were both associated to fungal diversity, highlighting the
491 contribution of the traits mass ratio as additional drivers of fungal diversity. Decreasing mean
492 phenolic concentration of litter mixtures (i.e. CWM1) was related to higher turnover rate of the
493 fungal community, affecting fungal diversity until 250 days of decomposition. This suggests
494 that fungal communities are more sensitive to phenolics than bacterial communities.
495 Interestingly, increasing mean C:N ratio value of litter mixtures (i.e. CWM2) was related to
496 higher fungal diversity after 50 and 100 days of decomposition. The C:N ratio of bacterial

biomass is expected to vary between 3 and 6 while the C:N ratio of fungal biomass is expected to vary between 5 and 15 (McGill et al., 1981). Subsequently, fungal communities are expected to have lower N requirement than bacteria (Güsewell and Gessner, 2009), and thus, being less N limited, they would be able to colonize and develop on poor-quality litter at early decomposition stages.

5. Conclusion

Our results from a field experiment clearly demonstrated that potential shifts in plant species diversity and associated litter traits may have strong cascading impacts on microbial decomposer communities in a Mediterranean oak forest. Both bacterial and fungal communities were influenced by litter species diversity, litter identity and litter functional traits. However, the intensity and the direction of these effects varied strongly during decomposition. Litter diversity effects were mediated by litter species composition rather than litter species richness, highlighting the importance of litter species identity (and associated litter traits) as driver of microbial communities. We also underscored the importance of *Quercus pubescens* (i.e. the dominant tree species of the studied oak forest) and *Pinus halepensis* (i.e. an alien tree species that may become more frequent in the studied oak forest in response to climate change) in structuring microbial communities. Both community weighted mean (CWM) litter traits (mass-ratio hypothesis) and functional dissimilarity (FD) of litter traits (niche complementarity hypothesis) contributed to litter diversity effects, with a greater relative importance of FD compared to CWM, and with an overall stronger impact on fungal compared to bacterial communities. Collectively, these findings also suggest that different aspects of litter diversity control microbial communities during litter decomposition.

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TABLES

Table 1 Effects of litter diversity (decomposed in species richness and species composition), time of litter decomposition and block on Shannon diversity index for bacterial and fungal decomposer communities. d.f. = degrees of freedom, %SS = percentage of sums of squares. *F*-values and associated *P*-values (with the respective symbols * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$) are indicated

	Bacterial diversity			Fungal diversity		
	d.f.	%SS	*F*-value	d.f.	%SS	*F*-value
Richness	3	1.5	**3.9****	3	5.9	**30.6*****
Composition	11	8.8	**6.5*****	11	8.8	**12.4*****
Time	5	33.2	**53.5*****	5	44.5	**138.1*****
Block	3	0.6	1.6	3	0.1	0.3
Richness × Time	15	4.9	**2.6****	15	3.0	**3.1*****
Composition × Time	55	20.4	**3.0*****	55	22.3	**6.3*****
Residuals	247	30.7		239	15.4	

Table 2 Effects of the presence of each litter species, time of litter decomposition and block on Shannon diversity index for bacterial and fungal decomposer communities. d.f. = degrees of freedom, %SS = percentage of sums of squares. *F*-values and associated *P*-values (with the respective symbols * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$) are indicated

	Bacterial diversity			Fungal diversity		
	d.f.	%SS	*F*-value	d.f.	%SS	*F*-value
Acer	1	0.6	3.4	1	0.0	0.0
Cotinus	1	0.3	1.8	1	0.0	0.1
Pinus	1	1.2	**7.4****	1	11.9	**119.6*****
Quercus	1	3.2	**19.1*****	1	1.5	**15.0*****
Time	5	32.8	**38.7*****	5	44.5	**89.6*****
Block	3	0.5	1.0	3	0.1	0.2
Acer × Time	5	2.5	**2.9***	5	0.9	1.8
Cotinus × Time	5	2.4	**2.8***	5	2.5	**5.0*****
Pinus × Time	5	2.7	**3.2****	5	8.2	**16.4*****
Quercus × Time	5	1.7	2.0	5	0.8	1.6
Residuals	307	52.0		299	29.7	

766 **Table 3** Effects of community weighted mean traits (CWM), functional trait dissimilarities
767 (FD), time of litter decomposition and block on Shannon diversity index for bacterial and fungal
768 decomposer communities. CWM1 and CWM2, and FD1 and FD2 represent the two first
769 components of the PCAs conducted using the CWM or the FD values in Fig. 4. d.f. = degrees
770 of freedom, %SS = percentage of sums of squares. *F*-values and associated *P*-values (with the
771 respective symbols * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$) are indicated
772

	Bacterial diversity			Fungal diversity		
	d.f.	%SS	*F*-value	d.f.	%SS	*F*-value
CWM1	1	0.3	1.4	1	5.1	**47.5*** **
CWM2	1	0.0	0.2	1	0.0	0.4
FD1	1	0.3	1.5	1	0.0	0.3
FD2	1	2.2	**9.8** **	1	11.3	**105.5*** **
Time	5	43.6	**39.6*** **	5	50.6	**94.3*** **
Block	3	0.1	0.6	3	0.0	0.2
CWM1 × Time	5	2.1	1.9	5	2.7	**5.1*** **
CWM2 × Time	5	1.1	1.0	5	1.5	**2.8***
FD1 × Time	5	0.2	0.2	5	0.2	0.4
FD2 × Time	5	3.1	**2.8***	5	6.4	**11.9*** **
Residuals	213	46.9		206	22.1	

773
774

775 **Table 4** Effects of community weighted mean traits (CWM) and functional trait dissimilarities
776 (FD) on bacterial and fungi community turnover rates during leaf litter decomposition. Adjusted
777 R^2 values in simple linear regressions and associated *P*-values (with the respective symbols *
778 for $P < 0.05$ and ** for $P < 0.01$) are indicated. The sign of the regression coefficient is indicated
779 in brackets. bCTR= bacterial community turnover rate; fCTR= fungal community turnover rate;
780 ns= non-significant relationship
781

	bCTR			fCTR		
CWM1	0.01	(+)	ns	0.38	(+)	*
CWM2	0.02	(-)	ns	0	(+)	ns
FD1	0.25	(+)	ns	0.01	(+)	ns
FD2	0.53	(+)	**	0.42	(-)	*

782
783

FIGURE LEGEND

Fig. 1 Bacterial (a, b, c) and fungal (d, e, f) diversity (expressed as Shannon diversity index H') according to time of decomposition (a, d), litter species richness (b, e) and litter species composition (c, f). Values are means ± SE (n = 60 for each decomposition time; n = 96, 144, 96 and 24 for 1-, 2-, 3-, and 4-species litter mixtures, respectively; n = 24 for each litter species composition). Different letters denote significant differences between treatments with a<b<c<d<e<f<g. A = *Acer*, C = *Cotinus*, P = *Pinus*, Q = *Quercus*. Combinations of capital letters correspond to combinations of plant species in litter mixtures

Fig. 2 Effects of litter species richness on bacterial (a, b, c, d, e, f) and fungal (g, h, i, j, k, l) diversity (expressed as Shannon diversity index H') according to time of decomposition. Values are means ± SE (n = 16, 24, 16 and 4 for 1-, 2-, 3-, and 4-species litter mixtures at each decomposition time, respectively). Different letters denote significant differences between treatments with a<b<c

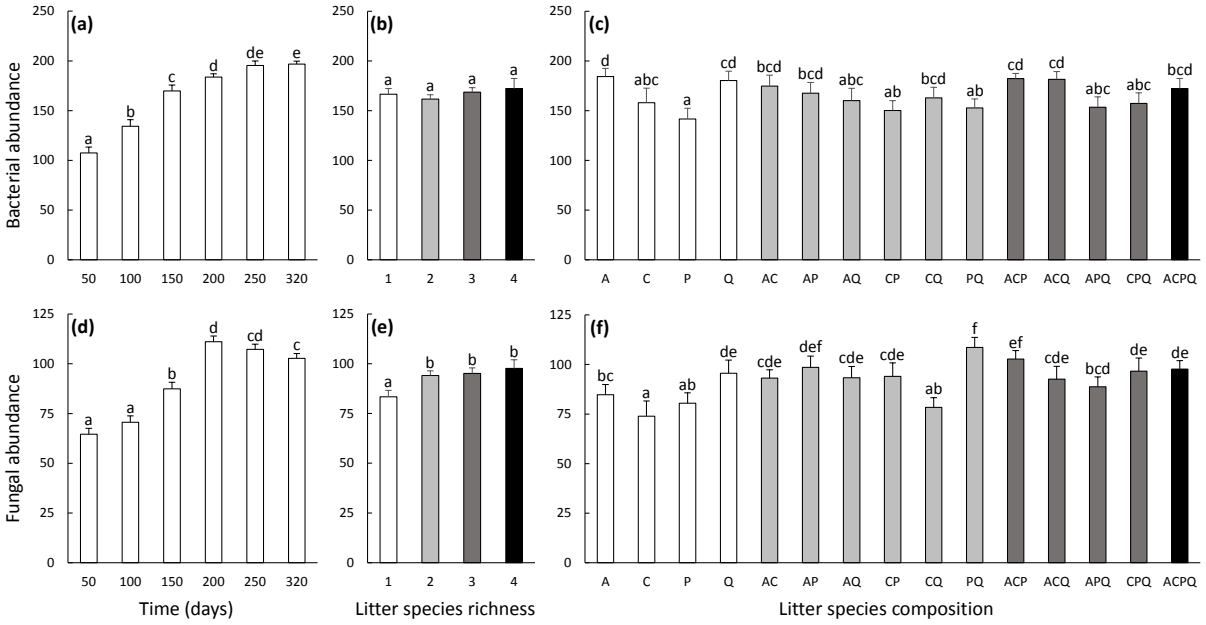
Fig. 3 Bacterial (a, b, c, d) and fungal (e, f, g, h) diversity (expressed as Shannon diversity index H') according to the presence of *Acer* (a, e), *Cotinus* (b, f), *Pinus* (c, g) and *Quercus* (d, h). Values are means ± SE (n = 32 and 28 for species presence and absence at each decomposition time, respectively). Presence = grey bar and Absence = white bar. Microbial diversity significantly different according to the presence/absence of litter species is indicated with the respective symbols * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$

Fig. 4 Principal component analysis (PCA) of community-weighted mean traits (a) and functional trait dissimilarity (b) based on the litter traits of the 11 multiple-species litter

809 mixtures. Variance explained by each principal component and associated eigenvalues are
810 shown in brackets. A = *Acer*, C = *Cotinus*, P = *Pinus*, Q = *Quercus*. Combinations of capital
811 letters correspond to combinations of plant species in litter mixtures
812
813 **Fig. 5** Bacterial (white symbol) and fungal (grey symbol) diversity (expressed as Shannon
814 diversity index H') as a function of functional trait dissimilarities (FD2 values; Fig. 4). Each
815 symbol represents the mean value (n = 4) of each of the 11 multi-species litter mixtures.
816 Significant linear relationships are indicated with dotted (bacteria) or solid (fungi) lines,
817 adjusted R^2 and associated *P*-values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). BD = Bacterial
818 diversity; FD = Fungal diversity
819

820 **Fig. 1**

821



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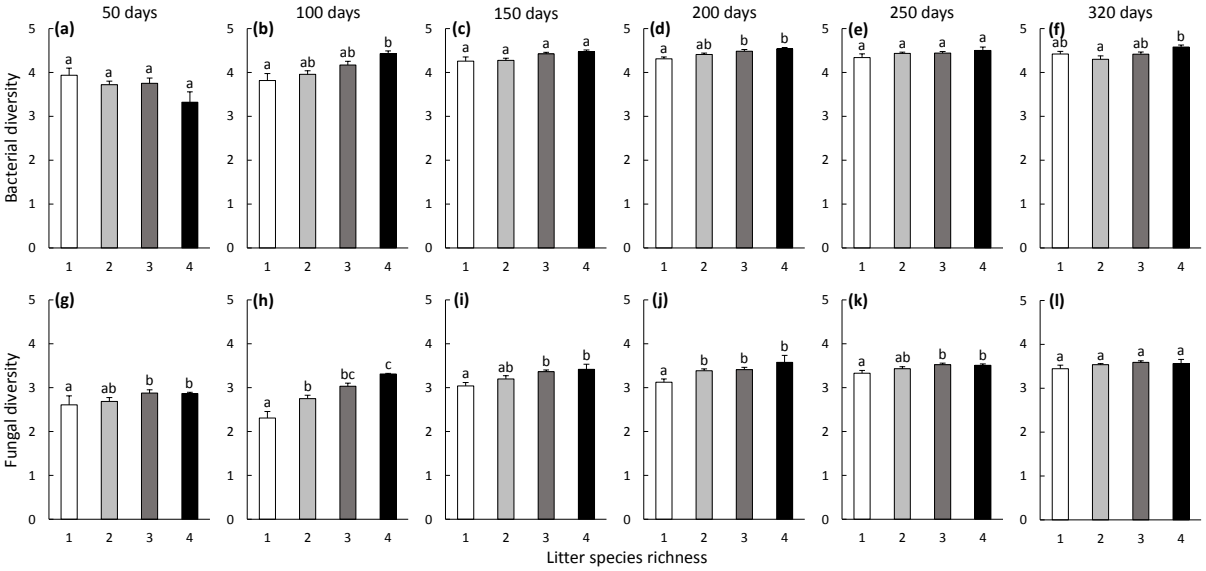
824 **Fig. 2**

825



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828 **Fig. 3**

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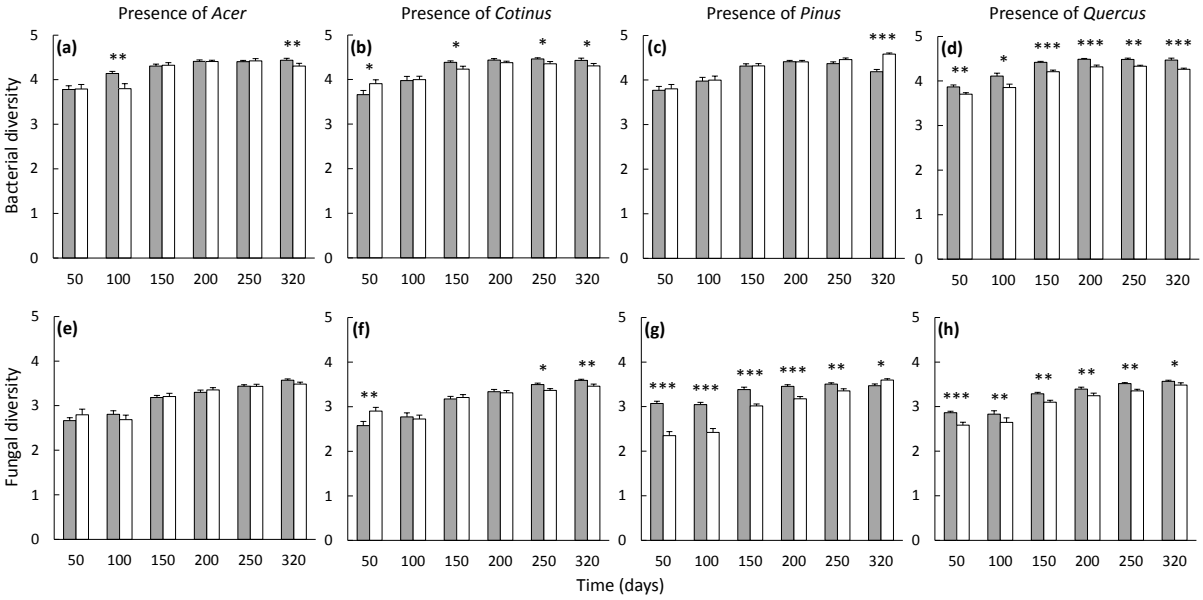

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832 **Fig. 4**

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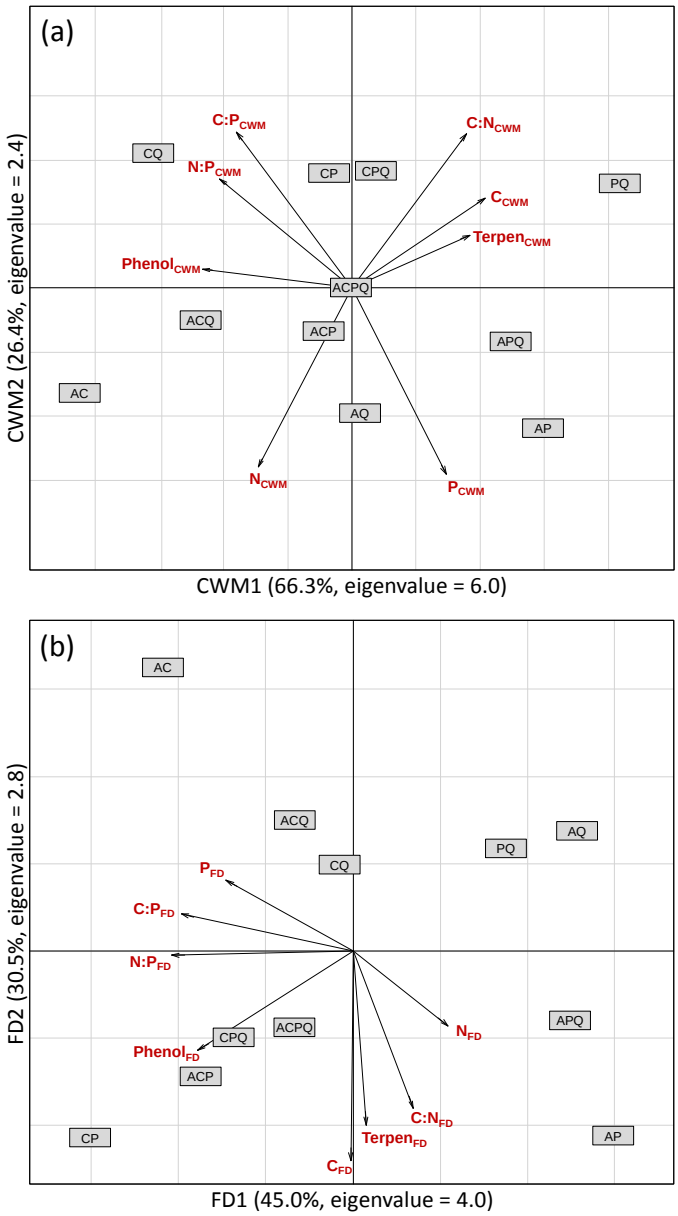

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836 **Fig. 5**

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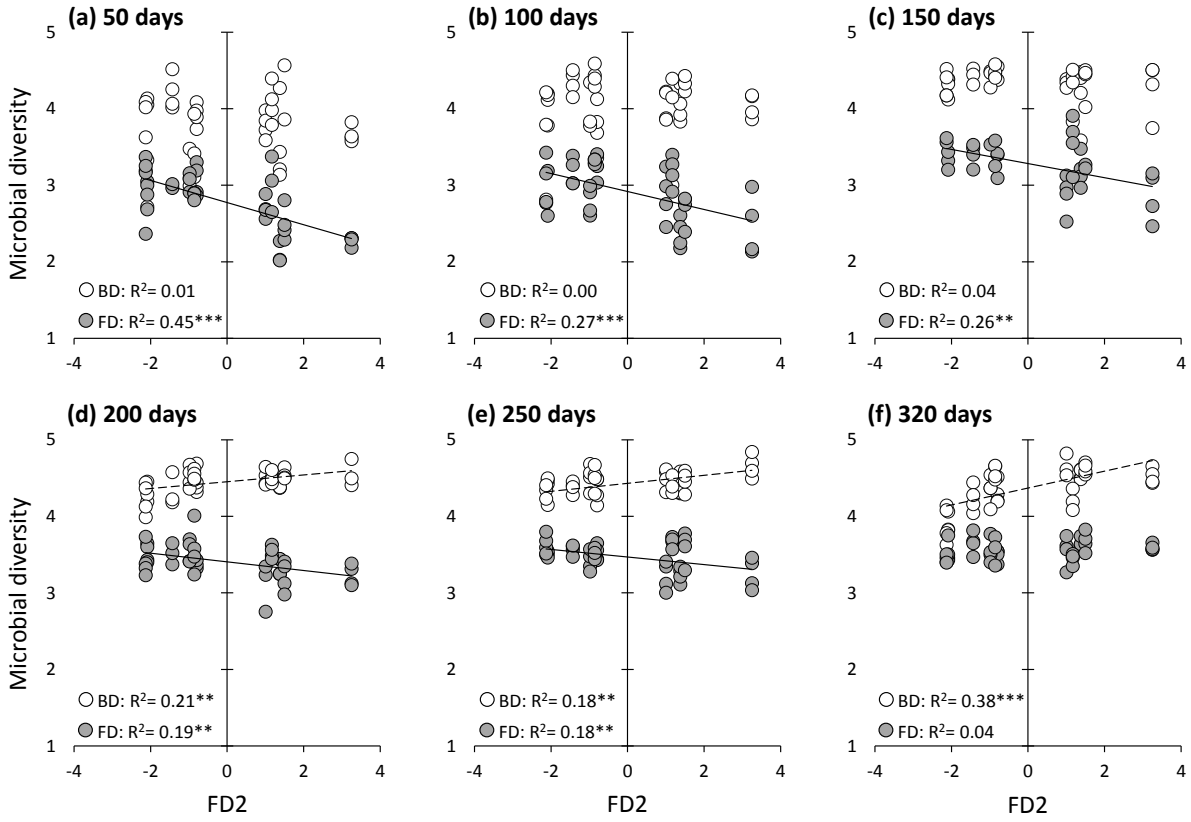

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