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REGULAR ARTICLE

Holm oak decline triggers changes in plant succession and microbial communities, with implications for ecosystem C and N cycling

Alexandra Rodríguez · Jorge Curiel Yuste · Ana Rey · Jorge Durán · Raúl García-Camacho · Antonio Gallardo · Fernando Valladares

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Abstract
Background and aims The occurrence of drought-induced forest die-off events is projected to increase in the future, but we still lack complete understanding of its impact on plant-soil interactions, soil microbial diversity and function. We investigated the effects of holm oak (*Quercus ilex*) decline (HOD) on soil microbial community and functioning, and how these effects relate to changes in the herbaceous community.
Methods We selected 30 holm oak trees with different defoliation degrees (healthy, affected and dead) and analyzed soil samples collected under the canopy (holm oak ecotype) and out of the influence (grassland ecotype) of each tree.
Results HOD increased potential nitrogen (N) mineralization and decreased inorganic N concentrations. These results could be partially explained by changes in the herbaceous composition, an increased herbaceous abundance and changes in soil microbial functional diversity and structure, with HOD favoring bacteria against fungi. Moreover, herbaceous abundance and microbial functional diversity of holm oak and grassland ecotypes converged with HOD.
Conclusions Our results show that HOD triggers a cascade effect on plant understory and soil microbial communities, as well as a plant succession (savannization) process, where understory species colonize the gaps left

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A. Rodríguez · J. Curiel Yuste · A. Rey · F. Valladares
Museo Nacional de Ciencias Naturales, MNCN, CSIC, 28006 Madrid, Spain

A. Rodríguez · J. Durán · F. Valladares
LINCGlobal, Madrid, Spain

J. Durán
Centro de Ecologia Funcional, CEF, Universidade de Coimbra, 3000-456 Coimbra, Portugal

R. García-Camacho · F. Valladares
Área de Biodiversidad y Conservación, ESCET, Universidad Rey Juan Carlos, 28933 Móstoles, Madrid, Spain

A. Gallardo
Departmento de Sistemas Físicos, Químicos y Naturales, Universidad Pablo de Olavide, 41013 Seville, Spain

Present Address:
A. Rodríguez (✉)
Centro de Ecologia Funcional, CEF, Departamento de Ciências da Vida, Universidade de Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal
e-mail: arp@uc.pt
e-mail: xandrouva@gmail.com

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by dead holm oaks, with important implications for ecosystem C and N budgets.

Keywords *Quercus ilex* · Forest die-off · C cycling · N cycling · Fungi · Bacteria · Herbaceous community

Introduction

Drought-induced forest die-off, triggered by increasing dry and hot climatic conditions, has been occurring on every vegetated continent over the past two decades (Allen et al. 2010, 2015; Anderegg et al. 2013). This syndrome can transform regional landscapes affecting stand structure and dynamics (McDowell 2011; Royer et al. 2011), as well as ecosystem carbon (C), and energy and water balances at large geographic scales (Martínez-Vilalta et al. 2012). However, the effects of drought-induced forest die-off on key aspects of the ecosystem, such as soil microbial community and functioning (Curiel Yuste et al. 2012; Lloret et al. 2014), nutrient cycling (Xiong et al. 2011) and understory plant communities (Anderegg et al. 2012) are still far from being understood.

Soil microorganisms are responsible for the majority of organic matter decomposition (Nielsen et al. 2011; Crowther et al. 2015), making a considerable contribution to soil respiration (Rey et al. 2002; Barba et al. 2016) and nutrient cycling (Wardle 1998; Allison and Martiny 2008), ultimately affecting the performance of plant communities and ecosystems (van der Heijden et al. 2008). On the other hand, plants strongly influence soil microbial communities and functioning by modifying environmental conditions, and providing microbes with exudates and litter (Fontaine et al. 2007; Prescott 2010; Barba et al. 2015). Tree mortality is therefore likely to directly affect microbial communities. Indirect effects may also occur through changes in the understory community, which is particularly vulnerable to tree mortality due to its high sensitivity to microclimate and low colonization capacity (De Frenne et al. 2011). Whereas the importance of soil microbial diversity and structure for the maintenance of ecosystem processes has been recently recognized (van der Heijden et al. 2008; Wagg et al. 2014; Delgado-Baquerizo et al. 2016; Graham et al. 2016; van der Plas et al. 2016), we still lack a complete understanding of how soil microbial community and functioning is affected by forest die-off (Curiel Yuste et al. 2012; Lloret et al. 2014).

Terrestrial C and nitrogen (N) cycles are tightly linked through ecosystem processes such as soil respiration and organic matter decomposition and mineralization (Finzi et al. 2011), but disturbances may decouple these cycles (Evans and Burke 2012). As forest soils store an enormous proportion of the potentially volatile global C pool (787 Pg C; Crowther et al. 2015), recent studies investigating the impacts of drought-induced forest die-off on microbial functioning have focused on C cycling (Curiel Yuste et al. 2012; Lloret et al. 2014; Barba et al. 2016). These studies suggest that die-off episodes could immediately curtail root and mycorrhizal respiration and reduce exudate supply from roots to soils, but also stimulate decomposition of litter, roots and dead wood (Nave et al. 2011). Evidence suggests that forest die-off could also trigger important changes in the N cycle, such as increased N mineralization rates followed by rapid N losses (Jenkins et al. 1999; Xiong et al. 2011; Edburg et al. 2012). However, despite the important consequences that these changes in N cycling could have on the nutritional status and the C sequestration capacity of ecosystems (Rodríguez et al. 2014), the effect of drought-induced forest die-off on the N cycle remains largely unexplored.

Mediterranean holm oak (*Quercus ilex*) forests have been lately suffering remarkable drought-induced tree defoliation and mortality (Lloret et al. 2004; Carnicer et al. 2011). This problem is likely to be exacerbated in the coming decades, since drought events are expected to become more frequent and intense in the Mediterranean region (IPCC 2013; Valladares et al. 2014). In monospecific forests, die-off could disproportionately affect plant community structure and dynamics (Anderegg et al. 2012; Allen et al. 2015), as well as ecosystem processes and functions (Xiong et al. 2011; Lloret et al. 2014). Moreover, repeated drought-induced mortality events, along with unsuccessful recruitment of the affected dominant tree species, may drive holm oak forests to undergo a plant succession process where *Q. ilex* would be replaced by understory species (Lloret et al. 2012; Saura-Mas et al. 2014; Ibáñez et al. 2015), with important implications for both soil microbial communities and ecosystem functioning.

We investigated the effect of drought-induced holm oak decline (hereafter HOD) on soil microbial community and functioning and its relationship with potential changes in the herbaceous community. We measured

different aspects of soil biogeochemistry and microbial functioning related to C and N cycling (pH, moisture, total C and N, inorganic N, potential N and C mineralization), estimated soil microbial community biomass and functional (alpha and beta) diversity and studied the abundance, diversity and composition of the herbaceous community. All variables were measured in soils under the canopy of holm oak trees with different degrees of defoliation (holm oak ecotype) and under the grassland outside of the influence of the holm oak trees (grassland ecotype). We hypothesized that HOD accelerates C and N mineralization and changes microbial composition and functional alpha- and beta-diversity, and that these changes are driven by associated changes in the herbaceous community. As the factors controlling soil microbial diversity and activity would likely change if grassland species replaced holm oak trees (Tang and Baldocchi 2005; Baldocchi et al. 2006), we also assessed the importance of different variables determining the soil microbial functional alpha-diversity and functioning (potential C and N mineralization) in the two ecotypes (holm oak vs. grassland).

Material and methods

Study site

The study was carried out in a holm oak forest located in the central part of the Iberian Peninsula, southwest of Madrid (40°23'N, 4°11'W; 630–660 *m* above sea level). The climate is continental Mediterranean with mean annual temperature and precipitation of 15 °*C* and 558 *mm*, respectively (Ninyerola et al. 2005). Most of the rainfall concentrates from autumn to spring, while summers are warm and dry. Soil is sandy (83–85% sand, 4–8% clay, 7–13% silt), upon fractured bedrock mainly composed of biotite granites and is classified as Cambisol (Monturiol and Alcalá del Olmo 1990). Tree density is ~180 trees ha^{-1}. Aboveground vegetation is mostly composed of *Quercus ilex ssp. Ballota* L. (holm oak) with scarce *Juniperus oxycedrus* Sibth. & Sm (cedar). The understory is dominated by *Retama sphaerocarpa* L., *Lavandula stoechas ssp. Pedunculata* (Mill.) Samp. ex Rozeira, and diverse pasture species, dominated by therophytes, being *Vulpia sp.*, *Bromus sterilis* L., *Xolantha guttata* L., *Hypochaeris glabra* L. and *Bromus tectorum* L. the most common species. One of the last severe droughts in the region occurred in 2005, a particularly dry year in the Iberian Peninsula (European Environment Agency 2008), with an annual rainfall of 251 *mm*, 55% lower than the average annual rainfall for this region (Getafe [40°17'N 3°43'O] and Cuatro vientos [40°22'N 3°47'O] stations, AEMET). A monitoring study revealed that this extreme drought resulted in a strong event of holm oak defoliation (around 20–30% of the total population) and mortality (15%) that remained until 2013 (F. Valladares, unpubl. data).

Experimental design

The effects of holm oak decline (HOD) on soil microbial community and functioning were analyzed considering *Q. ilex* individual trees as the experimental unit. In spring 2013, in a 12 *ha* forest plot, we selected 30 independent holm oak trees based on its crown defoliation degree: 10 healthy (less than 10% of its crown defoliated), 10 affected (more than 50% of its crown defoliated), and 10 dead (the whole crown defoliated). Crown defoliation was estimated visually, as in other studies (Anderegg et al. 2012, 2013; Curiel Yuste et al. 2012; Saura-Mas et al. 2014; Barba et al. 2015, 2016). We selected trees of similar size (based on diameter at breast height [dbh] and height, Table 1) and as much isolated as possible from other trees to minimize unwanted confounding effects of neighboring trees.

For each tree we established a 5 *m* north-facing transect with 2 sampling points, one under the tree canopy, at 0.3 *m* from the trunk (rhizosphere of holm oak ecotype), and the other one 5 *m* away from the trunk (rhizosphere of grassland ecotype), out of the influence of the tree (Tang and Baldocchi 2005; Rodríguez et al. 2011). This paired-sampling point design allowed us to measure the effect of HOD while distinguishing it from other confounding factors, such as the particular environmental conditions under individual trees and its surrounding environment caused by the inherent spatial variability of soils and soil metabolic activity (Barba et al. 2013). Further, it allowed us to better describe plant-soil-microbe relationships under two different environments influenced by two plant functional types with contrasting life history and phenology. We chose trees located in areas of similar slope (average slope for healthy, affected and dead trees was 4.5°; 3.8° and 4.9°, respectively) to avoid potential confounding effects such as run-off or differential erosion. In spring (May) and summer (September) of 2013, one soil sample of

Table 1 Means (1SE) and one-way ANOVA results of tree-related variables. Statistically significant effects of defoliation are represented by bold *P* values. Different letters next to values within each variable represent significant differences among defoliation degrees ($P < 0.05$). Canopy = canopy diameter; dbh = diameter at breast height; CI = competition index

				ANOVA		
Variable	Healthy	Affected	Dead	*F*	*df*	*P*
Height (*m*)	4.54 (0.20)	3.81 (0.15)	3.82 (0.32)	3.18	2	0.057
Canopy (*m*)	5.97 (0.31) a	4.46 (0.30) b	4.46 (0.37) b	7.02	2	**0.003**
dbh (*cm*)	45.85 (3.86)	33.37 (6.63)	33.84 (5.17)	1.76	2	0.191
CI ($\times 10^{-3}$)	0.8 (0.4) a	5.5 (2.7) ab	8.7 (3.3) b	4.46	2	**0.021**

$n = 10$

5 *cm* (i.d.) was collected from the first 10 *cm* of the soil profile in each sampling point using a metal corer and then kept at 4 °*C* until analysis. Total C and N content, pH and microbial functional diversity variables were analyzed in the spring soil samples, whereas soil moisture, inorganic N, microbial biomass, and potential C and N mineralization were determined in both spring and summer soil samples. We measured soil moisture in the first 5 cm of the soil profile of all our sampling points biweekly or monthly (depending on the season) from May 2013 to May 2015 using a handheld TDR probe (Spectrum Technologies, Inc., Plainfield, IL, USA).

Tree-related variables

We measured tree height, diameter at breast height (dbh) and the canopy diameter of the 30 studied trees by using a clinometer, a dbh tape (considering the sum of the different stems), and averaging two perpendicular measurements of canopy diameter, respectively (Table 1). We also estimated, for each one of the holm oak individuals, the classic distance-dependent competition index between trees (Hegyi 1974), considering competing holm oaks and cedars indistinctly:

$$CI_i = \sum_{j=1}^{n} \frac{dbh_j \Big/ dbh_i}{\mathrm{dist}_{ij}}$$

where CI_i is the competition index for the subject tree *i*; dbh_j is the trunk diameter at breast height for the competitor tree *j*; dbh_i is the trunk diameter at breast height for the subject tree *i*; $dist_{ij}$ is the distance between the subject and the competitor tree; and *n* is the number of competitors within a 5.5 *m* radius. This distance was selected based on the low density of trees and the relatively high occurrence of competitors between 5 and 5.5 *m*.

Soil biogeochemical variables

Soil samples were sieved (2 *mm* mesh size), homogenized in field-moist conditions and analyzed for gravimetric moisture by oven-drying a subsample at 60 °C to constant mass. Soil total carbon (C) and nitrogen (N) content, as well as pH, were estimated for the spring soil samples by dry combustion with an elemental analyzer (LECO TruSpec CN) and using a soil-to-water ratio of 1:2.5 (*m/v*), respectively. Given the temporal stability of these variables, we did not repeat these measurements in summer.

Inorganic N was extracted both from spring and summer soil samples by shaking fresh soil subsamples (5 *g*) with 25 *ml* of 0.5 *M* K_2SO_4 for 1 *h* at 200 *rpm* in an orbital shaker and filtering the suspension through a 0.45 *mm* Millipore filter. Then, we used these extracts to colorimetrically determine the amount of NH_4^+-N and NO_3^--N as described by (Durán et al. 2009). Inorganic N was calculated as the sum of NH_4^+-N and NO_3^--N. Soil microbial biomass was estimated both from spring and summer soil samples by using a modification of the substrate-induced respiration (SIR) method (Anderson and Domsch 1978). This method consists in the measurement of the initial maximum respiratory response of samples, related with the current size of living microbial biomass, after amending them with an excess of glucose (Anderson and Domsch 1978). A subsample of 10 *g* of fresh soil was incubated for two hours (time for the initial maximum respiratory response before any increase in microbial biomass) at 30 °*C* with 0.50–0.52 *g* glucose kg^{-1} dried soil (amount of glucose necessary for eliciting the maximum

respiratory activity of our samples). Glucose was dissolved in distilled water and added as a solution (3.33 *mg* glucose ml^{-1}) that made all soils to be at ~40% of their water holding capacity (WHC). After a two-hour incubation, we measured the CO_2 release by placing each sample inside a 1 *L* jar with a lid connected to a portable, closed chamber, soil respiration system (EGM-4, PP systems, MA, USA) during 60 *s*. Then, we used the ideal gas law equation to convert and extrapolate the net CO_2 increase (*ppm*) to mass of C (*m*) in the headspace of the jar:

$$m = \frac{ppm \times P \times V \times M}{R \times T}$$

where *P* and *V* are, respectively, the air pressure (*ATM*) and the known headspace volume in the jar (*L*), *M* is the atomic weight of carbon ($g\ mol^{-1}$), *R* is the universal constant of gases (0.08206 $ATM\ L\ mol^{-1}\ K^{-1}$) and *T* is the temperature (*K*) at the measurement time.

Microbial C and N mineralization

Potential heterotrophic respiration (R_H) and N mineralization were measured throughout a 40-days laboratory incubation of 50 *g* of fresh soil in darkness under constant conditions of temperature (25 °*C*) and soil moisture (25% WHC), environmental conditions representative of these Mediterranean ecosystems. Soil water content was kept constant during the experiment by adding water to compensate for any water losses. This laboratory incubation was repeated twice, for spring and summer soil samples. Due to logistic constraints we reduced the number of samples to six out of the 10 soil samples for each ecotype (holm oak and grassland) and each defoliation degree (healthy, affected and dead).

We periodically measured R_H during the incubation by placing each sample inside a 1 *L* glass jar with a lid connected to our EGM-4 and measured the increase in CO_2 concentration inside the sealed jar over 60 *s* on days 1, 2, 5, 7, 12, 19, 26, 33 and 40. As described above, we used the ideal gas law equation to convert and extrapolate the built up of CO_2 within the closed-loop created between the headspace of the jar and the EGM-4 during the 60 *s* to R_H. Then, we calculated the potential C mineralization rate by interpolation of rates of R_H between measurement dates. Potential C mineralization was normalized both by dry soil mass ($mg\ C\text{-}CO_2\ kg^{-1}\ soil\ day^{-1}$) and by soil initial C content ($g\ C\text{-}CO_2\ kg^{-1}\ C\ day^{-1}$).

We estimated the potential N mineralization rate as the net increase in total inorganic N over the incubation period. To do so, we measured soil inorganic N (NH_4^+-N + NO_3^--N) before and after the incubation of the soil samples as described above. Potential net N mineralization rate was normalized both by dry soil mass ($mg\ N\ kg^{-1}\ soil\ day^{-1}$) and by initial C content ($mg\ N\ kg^{-1}\ C\ day^{-1}$).

Microbial functional diversity

We estimated the functional diversity of soil microbial communities (both bacteria and fungi) using community-level physiological profiles (CLPP) assessed with Biolog® EcoPlates™ (BIOLOG Inc., Hayward, CA) following Flores-Rentería et al. (2015). These analyses were performed in spring, when both soil microbes and plants reach their peak activity in Mediterranean systems (Gallardo et al. 2000; Curiel Yuste et al. 2011), from five out of the 10 soil sampling points per defoliation degree and ecotype. Plates were incubated at 28 °C in a humidity-saturated environment and darkness. Optical density (590 nm), which is indicative of carbon-source utilization, was measured and recorded every 24 *h* during 7 and 10 days for bacterial and fungal plates, respectively, using a Victor3 microplate reader (Perkin-Elmer Life Sciences, Massachusetts, USA). Optical density (absorbance) at the day the plate reached the asymptote was the value used in all posterior analyses (120 and 168 *h* for bacterial and fungal plates, respectively). These incubation times are similar to those used in other CLPP studies at comparable incubation temperatures (Classen et al. 2003; Flores-Rentería et al. 2015). The value of absorbance from each well was corrected by subtracting the blank well (inoculated, but without a substrate), reducing the influence of differences in initial inoculum densities on the generated CLPPs, thus improving comparisons among contrasting soil types (Classen et al. 2003). Subsequently, we averaged the three values for each individual substrate within a plate.

Functional alpha-diversity (local scale), i.e. how diversified the species are within a site, of both bacterial and fungal communities was evaluated through functional richness (*S*), Shannon index (*H'*), and evenness

(*E*). Shannon and evenness indexes were calculated as follows:

$$\text{Shannon}\left(H'\right) = -\sum_{i=1}^{s} \frac{n_i}{N} \times \ln\left(\frac{n_i}{N}\right) \quad \text{Evenness } (E) = \frac{H}{\ln S}$$

where n_i is the absorbance of a specific well (C substrate), N is the whole absorbance of the plate and S (richness) is the total number of C substrate catalyzed.

We also estimated the functional beta-diversity, i.e. how diversified the sites are in species composition within a region, by using multivariate dispersion (see below).

Herbaceous survey

In spring 2014, we performed a survey of pasture communities in the same sampling points for ecotype and defoliation degree used for C and N mineralization and soil microbial functional diversity determinations. This survey was done by using two 2.5 m^2 quadrats placed perpendicularly to the transect at both sites of each sampling point. In each quadrat, we identified all herbaceous species and their abundance. Values from both quadrats were summarized. Then, we calculated species richness (S_{herb}) as the number of different species, herbaceous abundance (A_{herb}) as the total number of herbs and the herbs Shannon index (H'_{herb}) (see above).

Statistical analyses

We tested differences in tree-related variables among defoliation degrees by performing one-way ANOVAs. Effects of defoliation degree and ecotype, and the interaction between them, on soil biogeochemistry, soil microbial functioning, microbial C substrate consumption and functional alpha-diversity, and herbaceous diversity were assessed using linear mixed-effects models. Tree identity was included as random factor to account for the possible non-independence of the two ecotypes (holm oak and grassland) selected for the same tree. Simultaneous tests for general linear hypotheses (Multiple Comparisons of Means: Tukey Contrasts) were performed to test for pairwise statistical differences between ecotypes for each defoliation degree. Subsequently, and due to the strong influence of the ecotype factor, the effect of the defoliation degree within each ecotype level was separately evaluated by one-way ANOVA. Tukey's HSD were used as post hoc test ($P < 0.05$).

Differences in community-level physiological profiles (CLPP) of soil microorganisms (bacteria and fungi) and in herbaceous composition between different defoliation degrees and ecotypes (location effect) were explored with nonmetric multidimensional scaling (NMDS) using the Euclidean distance measure after root square transformation of the data. Each ordination ran until the lowest global stress score was found; scores were sufficiently low (< 0.12) in all runs for data to be interpreted reliably in two dimensions. We then used permutational multiple analysis of variance (PERMANOVA, $\alpha = 0.05$) to assess whether treatment groupings apparent in NMDS plots were significantly different. Euclidian distance was chosen against Bray Curtis distance based on the stress scores. Nonetheless, both distance measures provided results highly comparable (data not shown). Multivariate dispersion was used as a measure of beta-diversity (dispersion effect), which calculates the average distance of group members to the group centroid or spatial median in multivariate space (Anderson et al. 2006). The same Euclidian dissimilarity matrix used to calculate NMDS was employed to estimate one centroid for each soil provenance (i.e. under holm oak and under grassland for healthy, affected and dead trees). As proposed by Warton et al. (2012), we checked for restrictions in the use of Euclidian distance measure to analyze dispersion effect (i.e. the mean variance plots in all cases followed approximately a line of slope cero).

We used a multimodel inference approach based on information theory and ordinary least-squares (OLS) regression to evaluate the relative importance of tree-related variables (defoliation degree, tree height and competence index), soil biogeochemical variables (pH, C:N ratio, inorganic N, SIR), soil microbial functional alpha-diversity (bacterial and fungal H' and S) and herbaceous abundance over the microbial functioning variables (C and N mineralization) measured in spring in both separated ecotypes (holm oak and grassland; Burnham and Anderson 2010). Separately, we also used this approach to test the relative importance of those tree-related variables, soil biogeochemical variables and herbaceous abundance over the microbial functional alpha-diversity and richness, according to the ecotype.

We used the statistical packages: R 3.1.1 (R Core Team 2014), to perform ANOVA and linear mixed-effects models (lmer function from lme4 package, Bates et al. 2015); Primer 6 and PERMANOVA + (PRIMER-E Ltd., Plymouth, UK) to carry out all NMDS and PERMANOVA; and SAM 4.0 (Rangel et al. 2010) to perform the multimodel inference.

Results

Tree-related and soil biogeochemical variables

Defoliation was significantly higher in individuals with higher competition index (Table 1) and had a significant effect on soil pH and soil C:N ratio for both ecotypes together and for the grassland ecotype separately (Table 2). Considering both seasons together, defoliation had a statistically significant effect on inorganic N of the holm oak ecotype ($\chi^2 = 6.05$, $P < 0.048$), which tended to be consistently higher under healthy than under affected and dead trees both in spring and summer (Fig. 1a and b), mainly due to higher amounts of soil NH_4^+-N (Table S1, supplementary material). Substrate induced respiration (SIR) of spring samples was significantly higher near (grassland ecotype) dead trees than near living trees (healthy and affected, Fig. 1c). Ecotype (holm oak vs. grassland) had a significant effect on most of the soil biogeochemical variables, considering defoliation degrees both together and separately (Table 2 and Fig. 1).

Microbial C and N mineralization

Defoliation did not have a significant effect on potential C mineralization nor in N mineralization rates on a dry mass basis (Table 3). However, we did find a significant defoliation effect in the spring samples when we expressed these rates on a soil carbon basis, with lower values of potential C mineralization near dead than near healthy trees and lower values of potential N mineralization under healthy than under affected trees (Table 3). Ecotype significantly affected potential C and N mineralization on a dry soil mass basis, with higher values in holm oak than in grassland samples regardless of the sampling season (Table 3). This ecotype effect disappeared (spring) or reversed (summer) when we expressed potential C and N mineralization per unit soil carbon (Table 3).

Microbial functional alpha- and beta-diversity

NMDS and PERMANOVA did not show a significant separation of bacterial and fungal community-level

Table 2 Means (1SE) and mixed model results of soil physico-chemical variables. Statistically significant effects of defoliation and ecotype, as well as significant interactions of both factors, are represented by bold *P* values. Different letters next to values within holm oak (H) or grassland (G) ecotype represent significant differences among defoliation degrees performed with one-way ANOVA ($P < 0.05$). Grey bars on the left denote a significant ecotype effect for the respective defoliation degree ($P < 0.05$)

					Mixed models		
Variable	Ecotype	Healthy	Affected	Dead	$P_{defoliation}$	$P_{ecotype}$	$P_{interaction}$
Soil moisture (%)							
Spring	H	3.52 (0.54)	2.53 (0.22)	3.06 (0.33)	0.754	**<0.001**	0.067
	G	1.42 (0.11)	1.83 (0.35)	1.80 (0.23)			
Summer	H	1.33 (0.15)	1.19 (0.22)	1.14 (0.14)	0.802	**<0.001**	0.218
	G	0.49 (0.08)	0.50 (0.06)	0.60 (0.07)			
pH	H	6.58 (0.11)	6.30 (0.10)	6.56 (0.10)	**0.011**	**<0.001**	0.765
	G	6.10 (0.05) a	5.91 (0.05) b	6.17 (0.06) a			
TC (%)	H	2.78 (0.44)	2.27 (0.21)	2.77 (0.31)	0.717	**<0.001**	0.314
	G	1.11 (0.09)	1.25 (0.14)	1.26 (0.14)			
TN (%)	H	0.22 (0.03)	0.17 (0.02)	0.21 (0.02)	0.078	**<0.001**	0.941
	G	0.11 (0.01)	0.10 (0.01)	0.11 (0.01)			
C:N	H	12.12 (0.38)	13.36 (0.43)	13.12 (0.51)	**<0.001**	**<0.001**	**0.032**
	G	9.82 (0.29) a	12.99 (0.53) b	10.80 (0.36) a			

$n = 10$

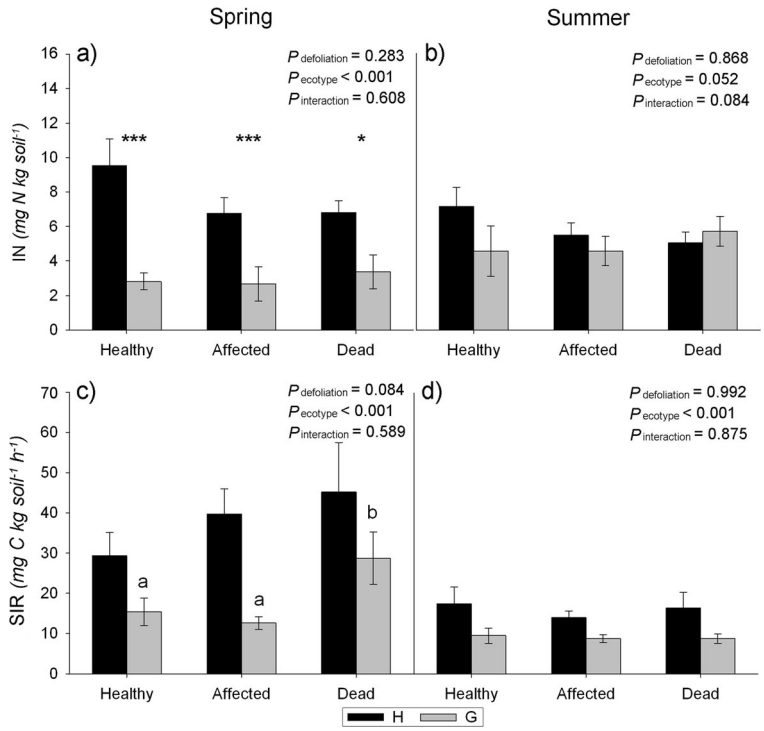

Fig. 1 **a-b** Inorganic N (IN) and (**c-d**) substrate-induced respiration (SIR) for spring and summer soil samples by defoliation degrees (healthy, affected and dead) and ecotypes [holm oak (H)-black bars; grassland (G)-grey bars], and results from mixed models. Bars and error bars represent means ($n = 10$) and standard errors of the mean, respectively. Different letters within each ecotype represent significant differences among defoliation degrees ($P < 0.05$). Asterisks indicate significant differences among ecotypes (* = $P < 0.05$; *** = $P < 0.001$)

physiological profiles (CLPP) by defoliation degrees considering both ecotypes together (Figure S1a and S1b). However, we found a significant defoliation x ecotype interaction on fungal CLPP (Fig. S1b), as well as on fungal alpha-diversity (H'_{fung}; Fig. 2d) and richness (S_{fung}; Fig. 2e). Under holm oak, H'_{fung} ($F = 5.55$, $P = 0.020$) and E_{fung} ($F = 5.82$, $P = 0.017$) significantly decreased as defoliation degree increased (Fig. 2d and f), while under grassland, S_{fung} ($F = 5.53$, $P = 0.020$) was significantly higher in samples collected near dead than near affected trees (Fig. 2e). Consequently, the ratio between bacterial and fungal alpha-diversity ($H'_{bact:fung}$; $F = 8.35$, $P < 0.005$) and evenness ($E_{bact:fung}$; $F = 6.37$, $P = 0.013$) was significantly higher under dead than under living trees (Fig. 2g and i). Considering all defoliation degrees together, we found significant differences between ecotypes both for soil bacterial and fungal CLPP (Fig. S1a-b), as well as for many of the microbial functional diversity parameters, which were significantly higher in the holm oak than in the grassland ecotype (Fig. 2a-c and e). Within each defoliation degree, we found significant differences between ecotypes for bacterial and fungal CLPP of living trees ($P < 0.05$), as well as for microbial functional alpha-diversity parameters of living trees, but never for those of dead trees (Fig. 2a-f). Substrate consumption was mainly dependent on the ecotype, although defoliation also affected the consumption of some substrates, particularly in the case of bacteria (Table S2 and S3). In general, we found higher values in holm oak than in grassland samples and under and near dead than under and near living trees (Tables S2 and S3).

Defoliation did not have a significant effect on microbial (bacterial and fungal) functional beta-diversity considering both ecotypes together (Fig. 3a-b). However, we did find a statistically significant defoliation x ecotype interaction and defoliation effect for holm oak and grassland ecotypes separately on the beta-diversity of the fungal community (Fig. 3b). Fungal beta-diversity was higher under dead holm oaks than under living trees, and lower near dead than near living holm oaks (Fig. 3b). Considering all defoliation degrees together, ecotype had a significant effect on the beta-diversity of both bacterial and fungal communities with lower values under holm oaks than under grassland (Fig. 3a-b). Within each defoliation degree, we found significant

Table 3 Means (1SE) and mixed model results of potential C and N mineralization rates measured during the 40-days soil laboratory incubation of spring and summer soil samples and normalized both on a dry soil mass and on a soil C basis. Statistically significant effects of defoliation and ecotype, as well as significant interactions of both factors, are represented by bold *P* values. Different letters next to values within holm oak (H) or grassland (G) ecotype represent significant differences among defoliation degrees performed with one-way ANOVA ($P < 0.05$). Grey bars on the left denote a significant ecotype effect for the respective defoliation degree ($P < 0.05$)

					Mixed models		
Variable	Ecotype	Healthy	Affected	Dead	$P_{defoliation}$	$P_{ecotype}$	$P_{interaction}$
Potential C mineralization							
mg C-CO_2 kg^{-1} soil day^{-1}							
Spring	H	93.2 (26.6)	52.7 (5.3)	82.6 (22.5)	0.491	**<0.001**	0.192
	G	34.2 (5.4)	37.1 (8.4)	21.3 (4.8)			
Summer	H	35.7 (7.1)	23.5 (7.3)	23.6 (5.8)	0.189	**0.009**	0.589
	G	21.1 (5.5)	15.1 (1.3)	15.2 (2.1)			
g C-CO_2 kg^{-1} C day^{-1}							
Spring	H	2.94 (0.64)	2.33 (0.26)	3.00 (0.76)	0.124	0.491	0.068
	G	3.24 (0.40) a	2.62 (0.40) ab	1.59 (0.31) b			
Summer	H	1.36 (0.29)	1.17 (0.47)	0.89 (0.17)	0.267	**0.023**	0.975
	G	2.26 (0.75)	1.23 (0.20)	1.19 (0.15)			
Potential N mineralization							
mg N kg^{-1} soil day^{-1}							
Spring	H	1.52 (0.09)	1.87 (0.15)	1.84 (0.20)	0.403	**<0.001**	0.070
	G	0.87 (0.10)	0.70 (0.06)	1.05 (0.21)			
Summer	H	1.73 (0.24)	1.82 (0.29)	1.74 (0.23)	0.637	**<0.001**	0.346
	G	1.31 (0.32)	0.91 (0.06)	1.30 (0.18)			
mg N kg^{-1} C day^{-1}							
Spring	H	58.8 (8.0) a	81.8 (4.3) b	72.1 (4.5) ab	0.786	0.534	**0.006**
	G	86.5 (14.0)	61.5 (8.7)	76.8 (6.0)			
Summer	H	69.3 (16.5)	82.9 (14.0)	69.7 (8.7)	0.818	**0.038**	0.120
	G	136.0 (43.6)	74.3 (13.3)	108.0 (24.9)			

$n = 6$

differences between ecotypes for the fungal beta-diversity of living trees, but not for those of dead trees (Fig. 3b).

Herbaceous community

NMDS and PERMANOVA showed a statistically significant separation of the herbaceous composition among defoliation degrees and ecotypes (Fig. S1c). Considering ecotypes separately, the holm oak ecotype showed significant differences among all defoliation degrees ($P < 0.05$) that disappeared in the grassland ecotype. Ecotype effect was statistically significant within each defoliation degree ($P < 0.05$). Mixed models showed a significant defoliation effect on herbaceous abundance (A_{herb}), with the highest values under and near affected canopies and the lowest values under healthy trees (Table 4). Ecotype had a statistically significant effect on herbaceous richness (S_{herb}) and A_{herb}, which showed in general higher values in the grassland than in the holm oak ecotype (Table 4). Considering defoliation degrees separately, the ecotype effect on A_{herb} remained for healthy and affected trees but disappeared for dead individuals (Table 4).

Predictors of microbial functioning and functional diversity

The multimodel inference tests revealed differences among the most important predictors of microbial functioning (potential C and N mineralization) and functional alpha-diversity, and between ecotypes (Table S4 and S5). Defoliation was the best predictor for some of the functional alpha-diversity variables, such as H'_{fung} of the holm oak ecotype (Table S4e) and S_{fung} of the grassland ecotype

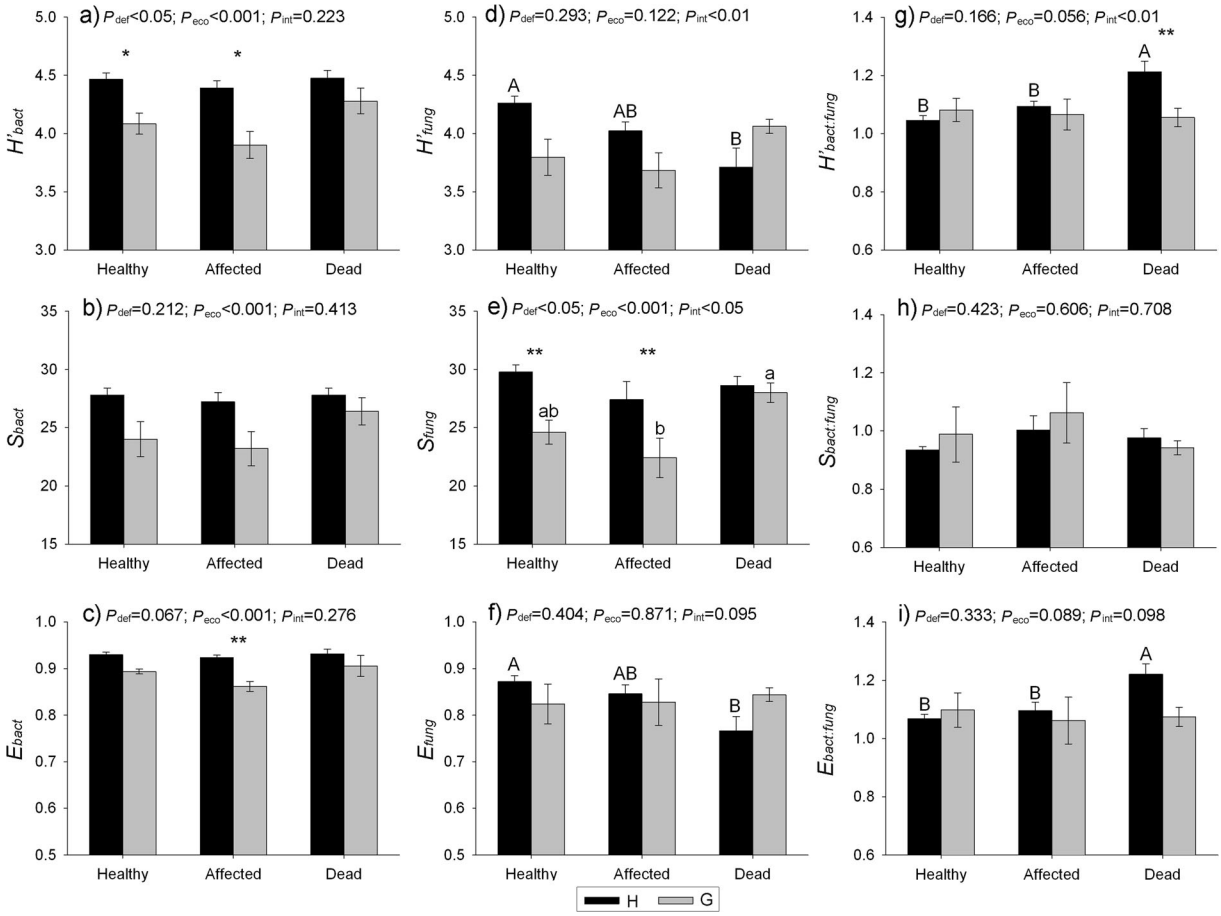

Fig. 2 Functional diversity parameters (Shannon index-*H*, richness-*S* and evenness-*E*) for (**a-c**) bacteria, (**d-f**) fungi and (**g-h**) ratio of bacteria:fungi by defoliation degrees (healthy, affected and dead) and ecotypes (holmoak [H]-black bars; grassland [G]-grey bars). Bars and error bars represent means (n = 5) and standard errors of the mean, respectively. P_{def}, P_{eco} and P_{int} show the significant level of the defoliation degree, the ecotype and the interaction of both, respectively (Mixed models). Different letters within each ecotype represent significant differences among defoliation degrees ($P < 0.05$). Asterisks indicate significant differences among ecotypes (* = $P < 0.05$; ** = $P < 0.01$)

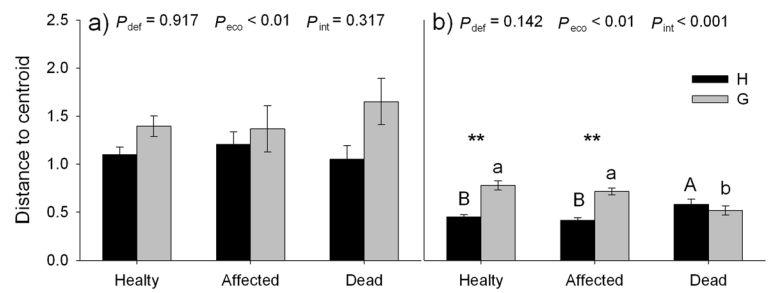

Fig. 3 Functional beta-diversity, determined by the distance to the centroid of multivariate dispersion, of (**a**) bacterial and (**b**) fungal communities of soils collected under the holm oak (H) and under the grassland (G) ecotype of the different defoliation degrees (healthy, affected and dead). Bars and error bars represent means (n = 5) and standard errors of the mean, respectively. Higher distance to centroid (over-dispersion) means higher beta-diversity. P_{def}, P_{eco} and P_{int} show the significant level of the defoliation degree, the ecotype and the interaction of both, respectively (PERMANOVA). Different letters within each ecotype represent significant differences among defoliation degrees ($P < 0.05$). Asterisks indicate significant differences among ecotypes (** = $P < 0.01$)

Table 4 Means (1SE) and mixed model results of herbaceous diversity variables. Statistically significant effects of defoliation and ecotype, as well as significant interactions of both factors, are represented by bold *P* values. Different letters next to values within holm oak (H) or grassland (G) ecotype represent significant differences among defoliation degrees performed with one-way ANOVA ($P < 0.05$). Grey bars on the left denote a significant ecotype effect for the respective defoliation degree ($P < 0.05$). H'_{herb} = herbaceous Shannon index; S_{herb} = herbaceous richness; A_{herb} = herbaceous abundance

					Mixed models		
Variables	Ecotype	Healthy	Affected	Dead	$P_{defoliation}$	$P_{ecotype}$	$P_{interaction}$
H'_{herb}	H	2.04 (0.21)	1.85 (0.43)	1.64 (0.29)	0.910	0.078	0.306
	G	1.95 (0.29)	2.40 (0.20)	2.31 (0.44)			
S_{herb} *(n° species)*	H	11.6 (1.4)	17.2 (3.7)	14.8 (3.2)	0.339	**0.005**	0.837
	G	18.2 (2.2)	22.0 (2.3)	18.8 (3.2)			
A_{herb} *(n° plants)*	H	104 (24) a	364 (76) b	265 (67) ab	**<0.001**	**<0.001**	0.257
	G	315 (42) a	760 (159) b	346 (27) a			

$n = 5$

(Table S5f), but never for microbial functioning variables. Similarly, A_{herb} was an important predictor for microbial (both bacterial and fungal) richness of both ecotypes (Table S4d, S4f and S5f), but not for microbial functioning variables. Tree-related variables, such as CI, were the best predictors for some of the holm oak ecotype models, such as potential N mineralization and H'_{bact} (Table S4b-S4c), but never for grassland ecotype models. Overall, models for the grassland ecotype were worse (lower explained variance [R^2]) than those for the holm oak ecotype.

Discussion

Impact of forest die-off on microbial functioning

Tree health (i.e. holm oak decline [HOD]) did not affect potential soil C mineralization (neither on a dry mass nor on a C basis), nor defoliation was an important predictor of potential soil C mineralization under holm oak trees (Table S4a). These findings, contrary to what we expected, suggest a certain resilience of soil microbial community in terms of their potential capacity to mineralize organic matter. Recent studies suggest that increased C inputs, soil moisture and N availability after die-off episodes should induce higher soil microbial respiration rates (Nave et al. 2011; Edburg et al. 2012). We did not measure C inputs, but the lack of increases in soil N availability (see below) and moisture with HOD could be in line with the lack of response in the potential C mineralization rates (Table 2). On the other hand, we acknowledge that potential instead of in situ estimations might have underestimated the effect of HOD on soil C mineralization.

Increased N mineralization and inorganic N availability after tree decline would be expected due to an altered soil microclimate, root death, and reduced demand for nutrients (Nave et al. 2011; Edburg et al. 2012). In our case, HOD did not affect potential N mineralization on a dry mass basis, but did it, positively, on a carbon basis, suggesting a switch to more efficient soil microbial communities (i.e. capable to mineralize more N per unit of carbon; Nielsen et al. 2011; Edburg et al. 2012) and/or a decrease in the litter C:N ratio (Nave et al. 2011). The observed HOD-driven switch to a more bacteria-dominated soil microbial community could partially support the faster N mineralization on a C basis (see below). Further, although the likely strong legacy of previous ecosystem conditions might have precluded us to find significant decreases in soil C:N with HOD, a decrease in the litter C:N ratio would be indeed expected after several years of defoliation and mortality due to decreases in leaf litter production and C-rich exudates, and increases in litter inputs from dead fine roots and mycorrhizas. Surprisingly, this increase in N mineralization was accompanied by an overall decrease in soil inorganic N. This apparent discrepancy could be at least partially explained by two complementary mechanisms. First, the reduction in competition for resources under the canopy of defoliated trees might have triggered increases in the herbaceous abundance (Table 4), as well as changes in their composition

(Figure S1c), such as the development of large-size species with high N requirements (e.g. *Bromus sterilis*; García et al. 2006). This changes in the herbaceous community might have helped to deplete the soil inorganic N, particularly in spring, whereas an increase in herbaceous litter with lower C:N ratio than that of holm oak would also agree with the above explained higher N mineralization rates per unit soil C. Second, the fact that the decrease in inorganic N with HOD was mostly due to a decrease in NH_4^+-N (Table S1), along with the switch to a more bacterial-dominated microbial community (see below), suggests that HOD could be favoring the autotrophic nitrification and therefore the presence of NO_3^--N, a highly mobile N form (Vitousek et al. 1979). Thus, it is likely that lower N and water tree uptake due to HOD, along with a more unprotected soil as a consequence of canopy defoliation, could have induced inorganic N losses through processes such as denitrification and leaching during rainfall events (Xiong et al. 2011; Nave et al. 2011). Indeed, the 2-years monitoring study carried out in the same site did show a significant increase (up to 63%) in the moisture of the first 5 *cm* of the soil profile with HOD (Table S6). However, we have to acknowledge that we did not find significant differences in the soil gravimetric moisture under different defoliation degrees, which suggest that our one-time per season measurements might provide an incomplete picture of these highly dynamic hydrologic and biogeochemical pools and processes.

We did not observe any change in total C, total N or the C:N ratio along the tree damage gradient (Table 2), and any extrapolation of our short-term study at individual-tree scale to the forest stand should be done with caution (Clark et al. 2016). However, the higher sensitivity to HOD of N- compared to C-cycling observed in our study, and the projected increases in forest die-off events clearly suggests that HOD might lead to a future decoupling of the C and N cycles. A higher sensitivity of N- compared to C-cycling to disturbances has already been observed and explained in terms of different sensitivities of ecosystem compartments and processes, such as the high susceptibility of N to be lost from the system, or the different sensitivity of soil microbial communities responsible for organic matter decomposition and N mineralization (Nave et al. 2011; Evans and Burke 2012; Durán et al. 2013; Morillas et al. 2015). Such decoupling could have major effects on the ecosystem, such as asynchronies in N supply and demand that, under certain abiotic conditions, would increase N losses and limitation (Evans and Burke 2012), with the consequent decrease in the capacity of forest to sequester C (Finzi et al. 2011; Fernández-Martínez et al. 2014; Rodríguez et al. 2014).

The differences among defoliation degrees observed for some of the biogeochemical and microbial functioning variables (i.e. pH, C:N, SIR, and total C mineralized on a C basis) in the grassland ecotype may suggest a HOD effect beyond 5 *m* from the tree trunk. However, the fact that these differences were not significant in the holm oak ecotype precludes this possibility. Alternatively, other factors such as the increased presence of competitors with holm oak decline, as evidenced by the competition index, could be at least partially responsible for the spatial heterogeneity observed in the grassland ecotype. Further, the observed increase in the competition index along the defoliation gradient, despite our efforts to select isolated individuals, supports that previous conditions, such as closed forest structures, might help spreading forest die-off events (Clark et al. 2016).

Impact of forest die-off on microbial functional diversity

Holm oak decline induced profound changes in the soil microbial community. First, we found a change in soil microbial community-level physiological profiles (CLPP) and a decrease in microbial functional alpha-diversity, but only for the fungal community. These results are in agreement with other studies showing that fungal communities are more sensitive than bacteria to forest die-off (Xiong et al. 2011; Lloret et al. 2014), largely explained by their association with roots and their function as specific woody debris decomposers (Coleman and Whitman 2005; Crowther et al. 2015). The strong effect of tree health on the functional diversity of fungal communities was also supported by the noteworthy importance of defoliation as the best predictor of fungal alpha-diversity parameters (Table S4e and S5f). Moreover, the significant decrease in the fungal functional evenness with HOD suggests that tree defoliation and mortality could influence not only the fungal functional diversity, but also the structure of the community, with a likely increase in the functional role of a limited number of dominant and/or rare taxa specialized in decomposing a reduced number of C sources (Lloret et al. 2014). Changes in soil microbial diversity, composition and structure (bacteria/fungi ratios) could have important implications in the ability of ecosystems to provide critical services (van der Heijden et al. 2008;

Wagg et al. 2014; Delgado-Baquerizo et al. 2016), beyond changes in microbial diversity alone (Nielsen et al. 2011). For instance, the HOD-triggered increase in the ratio of bacteria/fungi functional diversity and evenness, as well as in bacterial against fungal substrate consumption (Table S2 and S3), could decrease the capacity of the system to decompose less degradable soil organic matter (e.g. lignin) and sequester C (enhanced in fungi-dominated soils; Curiel Yuste et al. 2011; Lloret et al. 2014), as well as accelerate the nutrient cycling and subsequent nutrient losses from the ecosystem (faster in bacteria-dominated food webs; Moore et al. 2005; Xiong et al. 2011). Thus, changes in soil microbial community, favoring bacterial against fungal metabolism, could be at least partially behind the faster N mineralization on a C basis and the lower N availability with holm oak decline discussed in the previous section.

Surprisingly, whereas functional alpha-diversity (local scale) of the fungal community was significantly lower under dead than under healthy trees, we found the opposite pattern for the functional beta-diversity (landscape scale). A disturbance such as tree mortality may affect both alpha-diversity, via changes in environmental conditions, and beta-diversity, via changes in the spatial heterogeneity of those conditions (Maaß et al. 2014; Flores-Rentería et al. 2016). Our results indicate that, despite the fact that holm oak mortality might be associated with an impoverishment of the fungal functional diversity at local scale, it might favor habitat heterogeneity at landscape scale, causing a general enrichment of soil fungal functional diversity (Maaß et al. 2014; Flores-Rentería et al. 2016). This landscape-scale biotic heterogeneity could have even stronger positive effects on forest multifunctionality than those of local diversity, if species differ in the functions they support (van der Plas et al. 2016). In any case, our results point out the need to consider likely scale dependencies in the response of soil microbial communities to a disturbance to fully understand the implications of environmental perturbations on the ecology and functioning of soil microbial communities.

Drivers of microbial functioning and functional diversity

Not surprisingly, tree-related variables (e.g. CI), and variables closely related to tree production in holm oak forests (e.g. pH, Flores-Rentería et al. 2016) were more important in determining the rates of C and N mineralization under holm oaks than under grassland (Table S4 and S5). Established holm oak trees were able to increase the magnitude of soil biogeochemical and functioning properties, including those highly and poorly variable in time (Tables 2 and 3 and Fig. 1). The fact that the positive effect of trees on microbial activity disappeared (spring) or reversed (summer) when we expressed both potential C and N mineralization on a soil C basis indicates that, although higher, the organic matter content in the holm oak ecotype might be less labile (i.e. lower quality). This explanation would be consistent with the lower C:N values under grassland and with its herbaceous composition, mainly dominated by short-lived annual and rapidly degradable species. In addition, differences in the soil microbial communities between ecotypes could also help to explain these results. Indeed, in agreement with previous studies (Classen et al. 2003; Flores-Rentería et al. 2016), the presence of trees also changed the microbial CLPP. For instance, it exerted a strong positive influence on the substrate consumption (Table S2 and S3), the bacteria functional alpha-diversity (Shannon, richness and evenness) and the fungi functional richness. Interestingly, the influence of trees on CLPP turned negative at the landscape scale. The lower microbial functional beta-diversity in the holm oak than in the grassland ecotype indicates a biotic homogenization under the canopy of trees with respect to the open areas (Flores-Rentería et al. 2016), again highlighting the importance of considering the scale-dependency of the response of soil microbial communities to environmental changes.

Remarkably, when we considered dead individuals separately, all the differences in soil microbial functional (alpha and beta) diversity between ecotypes disappeared. These results imply a convergence in the soil microbial community of trees and grasslands both at the local and at the landscape scale with forest die-off. Similar results were reported by Curiel Yuste et al. (2012) in a mixed Mediterranean forest, and were explained by a colonization of the holm oak ecotype by grassland microbes after tree death. Moreover, the similar herbaceous abundance under grassland and under affected and dead trees, together with the importance of the herbaceous abundance in determining the soil bacterial and fungal functional richness in the holm oak ecotype (Table S4d and S4f), suggests that this convergence is tightly associated with the colonization by the herbaceous community. Thus, in agreement with Lloret et al. (2014) and supporting our hypothesis, our results

suggest that tree defoliation and mortality could initiate a cascade effect on plant understory and soil microbial communities, triggering a savannization process with profound changes in ecosystem C and N dynamics. The current mortality rate of our site is still relatively low (15%), so it is premature to insinuate the existence of a vegetation shift in the system (Lloret et al. 2012). However, the projected more frequent, hotter and longer drought events in an ecosystem structured by a single, strongly dominant, tree species could disproportionately affect key stabilizing mechanisms such as the recruitment of the dominant species (Allen et al. 2015). Under this scenario, in Mediterranean holm oak forests, the dominant *Q. ilex* will likely be replaced by smaller understory species, which would represent a major vegetation shift (Saura-Mas et al. 2014; Ibáñez et al. 2015). Whereas the differences among defoliation degrees in soils under holm oak trees indicate short-term responses (years), differences between ecotypes (trees vs. grassland) might indicate longer-term changes in ecosystem functioning (decades/centuries). Some of these long-term changes could be sharp reductions of the total C and N accumulated in the soil, decreases in the fungal functional diversity at the local scale, and increases in the fungal functional diversity at the landscape scale. The observed differences between ecotypes support that a successional replacement would imply not only a change in the landscape, but also in the functioning of the whole ecosystem (Barba et al. 2015, 2016), with clear implications for the ecosystem services provided by holm oak forests (Tang and Baldocchi 2005; Baldocchi et al. 2006).

Conclusions

Holm oak decline has a clear effect on soil microbial functioning and functional diversity and structure, which seems to be closely related to changes in the herbaceous abundance and composition. Microbial ability to cycle C appears to be resilient to holm oak decline, but our study shows evidences of a switch in the soil microbial community to one more capable to mineralize N, which could have important negative consequences for ecosystem N availability. The asymmetric response of potential C and N mineralization to HOD might anticipate a decoupling of those cycles in coming decades, and highlights the need for further research on the effect of forest die-off both at individual and ecosystem levels. The strong scale-dependency of the microbial functional diversity response to forest die-off, with contradictory effects on the fungal functional diversity at the local (negative) and landscape (positive) scale, stresses the complexity of the effects of environmental perturbations on soil microbial communities. Finally, the observed ecotype convergence in the herbaceous abundance and the microbial functional diversity in soils beneath dead trees suggests a likely HOD-driven plant succession process where *Q. ilex* would be replaced by understory species, with important implications for ecosystem features such as sharp reductions of the C and N budgets.

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