**Beneath the canopy: linking drought-induced forest die off and changes in
soil properties**

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Abstract

Climate warming and the occurrence of more severe dry spells are causing widespread drought-induced forest die-off events. Despite research on drought-triggered die-off processes is rapidly increasing, little is known on how soil conditions and rhizosphere features are affected by canopy dieback and tree death. We studied the soils in the rhizosphere of three coniferous forests where die-off was induced by a severe drought in 2012. We selected three forest types subjected to contrasting climatic and edaphic conditions dominated by three different tree species: silver fir (*Abies alba*; temperate conditions), Scots pine (*Pinus sylvestris*; continental and Mediterranean conditions) and Aleppo pine (*Pinus halepensis*; semi-arid and Mediterranean conditions). In each forest, we analyzed soil physical characteristics such as water retention capacity and soil texture, nutrient availability and microbial community structure (Phospholipid fatty acids, PLFA) below non-declining and declining or dying trees. We did not observe differences in nutrient availability between the two vigor classes. Conversely, we found strong differences in soil microbial community structure below non-declining and declining trees in the Silver fir and Aleppo pine stands. Soils in the Scots pine stand presented extremely low values of soil saturated sorptivity indicating a reduction of soil water infiltration after prolonged dry periods which could exacerbate drought stress. We conclude that forest dieback impacts the soil microbial community structure in the short term. Further research is required to understand the linkages between a reduced capacity of soil water infiltration after prolonged droughts, short-term changes in the soil microbiota, long-term nutrient imbalances and tree death. Soil conditions shall be considered as an important part of forest management strategies after drought-induced die off.

Keywords: forest dieback, PLFA, rhizosphere, soil hydrophobicity, soil microbial community structure, tree death.

1. *Introduction*

The reports of forest die-off events triggered by dry spells have increased considerably during the last decades (Allen et al., 2010). Such rising trend is of major concern for many forests worldwide if global warming amplifies drought stress (Anderegg et al., 2013; Allen et al., 2015; Camarero et al., 2015). The consequences of drought-induced forest die-off can be complex, acting at several temporal and spatial scales (Ruthrof et al., 2016), namely from the simple replacement of some trees to large compositional changes at the community level or even shifts in productivity at the ecosystem level due to the increase of soil surface temperature and decline in evapotranspiration (Royer et al., 2011; Anderegg et al., 2012). While catastrophic and widespread die-off events that cause the death of most trees have more drastic effects on forests, gradual die-off processes affecting some individuals, while other neighbouring trees survive, have less obvious consequences on tree-soil interactions. How soils will respond to the forecasted increase in drought and related forest die-off disturbances is a pivotal question in current global-change ecology (Curiel Yuste et al., 2011; Brunner et al., 2015; Phillips et al., 2016; Baldrian 2017).

Many ecosystem goods and services provided by forests depend on ecosystem processes such as carbon (C) and nitrogen (N) cycling which are part of forest belowground processes (van Der Heijden et al., 2008; Curiel Yuste et al., 2011; Baldrian 2017). For example, the C stock in forest soils exceeds by far that in aboveground vegetation (Jobbágy and Jackson, 2000), and it is estimated that 40-70% of the C photosynthetically assimilated by forests is transferred to the rhizosphere (Hopkins et al., 2013). Along these lines, the C balance between aboveground vegetation and soil microbial respiration is controlled by N supply which controls C uptake by plants (Thomas et al., 2015; Wurzburger and Brookshire 2017). Thus, nutrient cycles largely depend on the interaction between physicochemical soil properties, tree species identity and microbial communities.

The occurrence of abrupt disturbances such as forest die-off events caused by drought or pest outbreaks can have important influences on soil microbial communities and belowground nutrient cycling (Stursova et al., 2014; Pold et al., 2015). Pathogens causing forest die off (e.g. *Phytophthora* and *Heterobasidion* species) develop a substantial part of their life-cycles in the soil (Brasier et al., 1993; Oliva and Colinas 2007). In addition, soil physical and chemical properties such as low water-holding capacity largely affected by soil hydrophobicity (water repellence), and nutrient deficiency can exacerbate the negative consequences of drought stress on tree vigour leading to forest die-off (Doerr et al., 2000; Pinto and Peñuelas 2007; Hallett et al., 2011). However, these changes in soil conditions can arise because of forest die-off since tree species can modify soil organic matter content and water retention capacity (Lebron et al., 2007).

The influence of drought-related die-off on forest soil conditions may have different pathways. If phloem transport within declining trees is weakened, the composition of soil nutrients below the tree may change since C and N organic concentrations in the soil decrease (Dannenmann et al., 2009) while inorganic N concentration increases (Kreuzwieser and Gessler 2010). This has been shown by girdling experiments causing tree death and altering the quantity and quality of C and N compounds reaching the rhizosphere (Stursova et al., 2014; Pold et al., 2015). In dry regions, drought can also reduce phosphorous (P) and potassium (K) uptake by trees increasing their concentration in the soil (Sardans and Peñuelas 2007). If rhizodeposition, i.e. root exudates (organic compounds released into the soil by plant roots), is depressed then there may be a decrease of microbial biomass or activity in the rhizosphere (Dannenmann et al., 2009) or changes in the diversity of the soil microbiota (Schulze et al., 2005).

The processes involving the interaction between trees and soil microbes are very dynamic in space and time (Bahram et al., 2015; Baldrian 2017). For example, soil microbes

are able to utilize root exudates in minutes or few hours, but they will need several years to decompose dead wood (Baldrian 2017). The decline and death of a tree reduces or stops the supply of carbohydrates from the roots to the soil via root exudation (Brunner et al., 2015). However, the subsequent fall of dead needles and branches could feed soil microorganisms with different carbon sources through decomposition (Kana et al., 2012; Mikkelsen et al., 2016). However, the use of this readily available organic matter by microorganisms depends on the composition and activity of microbial communities as well as on environmental conditions (Pisani et al., 2014; Kuzyakov and Blagodatskaya, 2015). Thus, it is expected that drought-triggered forest die-off is anticipated to induce changes in soil microbial structure (Curiel Yuste et al., 2012; Stursova et al., 2014; Lloret et al., 2015). Nevertheless, it is unclear if these post-drought changes in the soil microbiota are similar among forests showing die-off but presenting different soil types and subjected to contrasting climate conditions.

Drought could also alter physical and chemical soil features (structure, water holding capacity, pH, hydrophobicity, nutrient availability) and decrease the enzymatic activity of the soil microbiota (Dannenmann et al., 2009; Curiel Yuste et al., 2011; Baldrian et al., 2013), leading to a negative feedback on tree nutrition by reducing nutrient mineralisation by bacteria (Kreuzwieser and Gessler, 2010). For instance, in *Eucalyptus* forest showing die-off this was associated with changes in the soil bacterial functional diversity linked to a decrease in the utilization of carbohydrates, amino acids and amines by the soil bacterial communities in sites with declining tree health (Cai et al., 2010). Furthermore, forest die-off usually leads to a reduction in canopy cover, which could increase the radiation reaching the ground and enhance evaporation further exacerbating soil dryness and contributing to more extreme microclimatic conditions in the uppermost soil, where most fine roots are found, thus reducing the rhizosphere microbiota activity (Sardans et al., 2008).

Tree species composition has important influences on forest soil physical and chemical characteristics (Augusto et al., 2015). As a consequence, there can be a large variation in soil microbial community composition between different forest types (Baldrian 2017). Drought may impact forest soil differently depending on the forest type studied and its prevailing climatic conditions (Aponte et al., 2013). For example, while climate warming may enhance soil enzyme activity in temperate hardwood forests of central Europe (Baldrian et al., 2013), it might have contrasting influences on drought-prone Mediterranean forests (Sardans and Peñuelas 2007; Sardans et al., 2008). However, the soil physical properties as well as soil nutrient content and microbial community structure may also vary at the scale of centimetre in forest soils (Koorem et al 2014; Nacke et al., 2016). Thus, forest soils may differ between neighbouring declining and non-declining trees growing in the same stand (Curiel Yuste et al., 2012; Mikkelsen et al., 2016).

Here we compare belowground soil properties (physical features, nutrient availability, microbiota composition) of coexisting declining or recently dead vs. non-declining trees in three forest types which experienced a drought-induced die-off in 2012. These forests are dominated by Scots pine (*Pinus sylvestris*), Silver fir (*Abies alba*) and Aleppo pine (*Pinus halepensis*), respectively, and they are subjected to contrasting climatic conditions. After the drought in 2012, we monitored the canopy cover, a proxy of tree vigour (Dobbertin 2005), of surviving trees. In 2015, we measured soil physical and chemical characteristics and characterized the soil microbial structure. We hypothesize that despite strong differences in nutrient supply rates and soil microbial structure linked to different forest characteristics (soil type, climate condition), die-off will be followed by a shift in nutrient availability and microbial community structure in the rhizosphere of declining and recently dead trees.

2. Materials and methods

Study sites and sampling protocols

We studied the populations of three conifers inhabiting three sites situated in Aragón (north-eastern Spain) and subjected to contrasting climatic conditions (Camarero et al., 2015). Particularly, we studied: a Scots pine (*Pinus sylvestris* L.) forest located in the Iberian System (Corbalán, Teruel) subjected to a continental Mediterranean climate (Table 1), a silver fir (*Abies alba* Mill.) forest situated in the Pyrenees (Paco Ezpela, Ansó) where temperate conditions prevail, and an Aleppo pine (*Pinus halepensis* Mill.) forest situated in the Middle Ebro Basin (Peñaflor, Zaragoza), close to the Monegros steppe, characterized by a semi-arid Mediterranean climate (see sites' features in Table 1). The Iberian system and the Pyrenees represent the southernmost distribution limit of Scots pine and Silver fir in Europe. The Ebro Basin is one of the driest regions in Europe. Climate warming and the occurrence of severe droughts during the late 20th century and early 21st century (1986, 1994-1995, 2005, 2012) have caused die-off events affecting the study forest types (Camarero et al., 2015; Vicente-Serrano et al., 2010; Supporting Information, Fig. S1).

The Corbalan Scots pine forest present no evident signs of human management during the last 50 years (Camarero et al. 2015). The Paco Ezpela silver-fir forest present signs that intense logging activity was undertaken in the past such as stumps and wood trails (Sangüesa-Barreda et al., 2015). Nevertheless, these management activities ceased in the early 1950s (Camarero et al., 2015). The Peñaflor Aleppo pine stands were part of a large recreational forest which in the past was reserved for the sole use of the aristocracy for hunting and recreation purposes. No evident signs of management were observed in this forest since the 1950s.

Soils were of the loam and loamy sand types in all sites (Supporting Information, Table S1). The soil pH varied from 6.79 in the Silver fir forest to 7.33 in the Scots pine forest and 7.77 in the Aleppo pine forest. The percentage of clay was higher in the Silver fir forest

170 than in the pine forests (13.22% more). Conversely, the percentage of sand was higher in the
171 Scots pine forest.

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176 **Table 1.** Main features of the three study conifer forests showing drought-induced forest die-off in northeaster Spain. The name of each site (and
 177 region), the dominant tree species, together with size features (diameter at breast height – dbh; tree height) of monitored trees (mean $\pm$ SD) and
 178 the percentage of dead trees in 2012 and 2015 are shown. MAT and TAP stand out for mean annual temperature and total annual precipitation,
 179 respectively (data correspond to the period 1950-2012).

Site (nearby locality)	Tree species	Latitude (N)	Longitude (W)	Bedrock	Soil type	MAT (°C)	TAP (mm)	Dbh (cm)	Height (m)	Mortality in 2012 (%)	Mortality in 2015 (%)
Corbalán (Teruel)	Scots pine (<i>Pinus sylvestris</i>)	40°26'	0°58'	Limestones	Cambisol, loamy	12.0	371	27.3 $\pm$ 1.3	8.3 $\pm$ 0.3	24	82
Paco Ezpela (Ansó)	Silver fir (<i>Abies alba</i>)	42°45'	0°52'	Marls, limestones	Cambisol, loamy	9.5	1153	36.7 $\pm$ 1.3	23.7 $\pm$ 0.6	37	47
Peñaflor (Zaragoza)	Aleppo pine (<i>Pinus halepensis</i>)	41°47'	0°44'	Marls, gypsum	Regosol, loamy	15.2	324	32.7 $\pm$ 1.6	8.1 $\pm$ 0.3	16	29

In each forest, an area between 0.5 and 2.0 has was sampled in 2012. We randomly selected 38 dominant trees separated at least by 5 m in each forest that were permanently marked and monitored. The year of death (last and complete tree-ring formed) of all recently dead trees was estimated using dendrochronology (Camarero et al., 2015). In the Scots pine forest, we selected 9 trees showing evident signs of decline (canopy dieback, abundant dead shoots and branches, needle shedding) and 29 trees displaying no evidence of decline (non-declining trees). In the silver-fir forest, we selected 14 trees showing evident signs of decline and 23 trees displaying no evidence of decline. In the Aleppo pine forest, we selected 6 declining trees and 32 non-declining trees. From 2012 to 2015, each site has been visited twice a year. Many trees considered declining in 2012 finally died during the 2012-2015 monitoring period (Table 1). In 2015, thirty-one Scots pines showed evident signs of canopy dieback and dead shoots; eighteen silver-fir individuals were dead; and eleven Aleppo pine trees were dead. Thus, for this study we considered declining or recently dead trees as those threes showing evident signs of decline in 2012 (more than 50% of crown transparency, abundant dead shoots and needles) and the one dying (no living needles) that year or during the period of monitoring (2012-2015). We considered non-declining trees as those trees that were marked as non-declining in 2012 and survived until 2015 and whose crown transparency was always lower than 50%.

2.1. Soil texture, saturated sorptivity, hydraulic conductivity and nutrient content

For several trees in each site, we collected three soil samples from the uppermost 25 cm, where most fine roots are found (Janssens, et al. 2002), around the trunk (less than 50 cm in distance) and below the canopy projection of each sampled tree. Prior to the collection of the samples the litter layer was removed carefully. In total, 26 trees were sampled in the Scots pine forest, 34 trees in the silver-fir forest, and 24 trees were sampled in the Aleppo pine

forest accounting for the same number of declining and non-declining individuals. Soil samples were stored in plastic bags and transported to the laboratory for their analyses. Samples for soil microbial structure analyses were frozen and stored in the freezer prior to the analyses. Specifically, 30 samples (3 samples per tree in 5 declining and 5 non-declining trees) were selected in each site for the analyses of soil microbial structure. Soil texture was determined with laser diffraction method in a Coulter Mastersizer 2000 and clay content was corrected following Taubner et al. (2009). Soil C and N concentrations were determined with an elemental analyzer (Elementar VarioMAX N/CM, Hanau, Germany).

Several soil samples were air dried during several weeks and sieved at 2-mm mesh size. In laboratory, the saturated sorptivity and (S) hydraulic conductivity (K_s) were estimated with a sorptivimeter device. The saturated sorptivity was calculated from the inverse analysis of an upward infiltration curve as described by Moret-Fernández et al. (2017), and K_s was measured using the Darcy's law from an overpressure step at the end of the upward infiltration processes. S defines the first steps of the soil water infiltration when the soil is dry, whereas K_s measures hydraulic conductivity once the soil column is saturated by water (Hallett et al., 2011). A total of 18 soil samples were analyzed (8 samples from the Silver fir site, which has more heterogeneity in tree vigor, corresponding to 6 non-declining and 2 declining trees; 5 samples from the Scots pine site corresponding to 3 non-declining and 2 declining trees; and 5 samples from the Aleppo pine site corresponding to 3 non-declining and 2 declining trees).

2.2. Soil nutrient supply rates

We established Plant Root Simulator (PRS) resin probes (Western Ag, Saskatoon, Canada) to measure the *in situ* availability to trees of several nutrients including N, P, K, other micronutrients (see for instance Harrison and Maynard 2014). In each forest, PRS resin

probes were located around the trunk of 5 declining and 5 non-declining trees (30 samples in total). The probes were placed in the uppermost 15 cm of the soil and less than 50 cm apart from the tree trunk and were kept in the field during the growing season (early April to late June). After this time, the probes were recovered and send to the manufacturing company for their analyses, providing an estimate of the available NO_3N , NH_4 , K, P, Ca, Mg, Fe, Mn, Cu, Zn, B, S, Pb, Al and Cd. These probes represent a non-destructive integrated way of obtaining the soil nutrient supply rate expressed as $\mu\text{g}/10\text{cm}^2/\text{burial period}$ (91 days in this study).

2.3. Soil microbial community structure

In each forest, we studied the microbial community structure in the soil surrounding the trunk of the 5 declining and the 5 non-declining trees in which the PRS resin probes were located. Phospholipid fatty acids (PLFA) can be used to monitor changes in microbial community structure as well as biomarkers for specific microbial groups. PLFAs were extracted from 2 g of lyophilized soil, separated and methylated following the methodology described by Buyer and Sasser (2012). The resulting fatty acids methyl esters (FAMES) were separated by gas chromatography using an Agilent 7890A GC System equipped with a HP-ULTRA 2 column (length 25 m, ID 0.20 mm; J&W Scientific Inc.) and a flame ionization detector. The individual FAME peaks were identified and quantified by the PLFAD1 method of Sherlock® software version 6.3 from MIDI, Inc (Newark, DE, USA). The internal standard 19:0 phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) was used for quantification of FAMES. Total microbial biomass was estimated by summing the contents of all individual PLFAs and reported as nanomoles of PLFAs per gram of soil. Specific PLFAs were used as biomarkers to quantify biomasses of eukaryotes, Gram-positive and Gram-negative bacteria,

actinobacteria, fungi, arbuscular mycorrhizal (AM) fungi and anaerobic bacteria (Frostegård and Bååth, 1996; Zelles, 1997; Zelles, 1999).

2.4. Statistical analyses

We used Generalized Least Square models (GLS; Venables and Ripley, 2002) to assess the differences in water retention capacity (the parameters K_s and S) between forests. We proposed two contrasting models: (i) one containing only the intercept as fixed factor and (ii) one containing forest as fixed factor. The intercept-only model represents no differences between forests. We selected the most parsimonious model as the one showing the lowest corrected Akaike Information Criteria (AICc). The inclusion of forest as fixed factor in the final model indicates that there exist differences between forest soils. The analyses were performed with the *nlme* package (Pinheiro et al., 2014) of the R statistical software (R development Core Team 2017).

A similar GLS procedure was used to study the differences in soil nutrient content (C, N content and C/N ratio), soil nutrient supply rate (PRS resin probes) and major groups of soil microorganisms (PLFA) between forests and between declining and non-declining trees. In this case, we proposed three contrasting models: (i) one containing only the intercept as fixed factor; (ii) one containing forest as fixed factor; and (iii) one containing the interaction between forest and vigour class (declining vs. non-declining) as fixed factors. As before, the intercept-only model represents no differences between forests. Competing models were ranked according to their $\Delta AICc$ and we also calculated the Akaike weight (W_i) of each model which measures the probability that the selected model is the best one. The inclusion of the interaction term indicates the existence of differences in soil variables between vigour classes within each forest. When a significant interaction between forest and vigour class was detected, we performed a separate Student's *t* tests for each site to compare declining and

non-declining trees. Soil nutrient content and soil nutrient supply rates for the different elements were log-transformed ($\log x+1$) if required to achieve normality assumptions prior to the analyses. The major groups of the microorganisms considered in these analyses were: the total biomass of microorganisms, Eukaryote, Gram negative and Gram positive bacteria, actinobacteria, saprophytic fungi and arbuscular mycorrhizal (AM) fungi. These groups, excluding the total biomass of microorganisms, are measured as proportions (i.e. they can only range between 0 and the total biomass of microbes found in the sample) so they were transformed prior to the analyses with the following additive log-ratio function (Aitchison 1982).

$$y = \ln \left(\frac{groupbiomass_i}{(totalbiomass - groupbiomass_i)} \right) \quad (1)$$

where $groupbiomass_i$ represents the abundance of the i group of microbes and $totalbiomass$ is the total amount of biomass of microbes found in the sample.

We used Non-Metric Multidimensional Scaling (NMDS; Legendre and Legendre 2012) with Euclidean dissimilarity to visualize patterns in soil nutrient supply rate (PRS resin probes). Similarly, we applied NMDS to visualize patterns in soil microbial community structure (PLFA). The NMDS axes of soil microbial community structure were correlated with the soil nutrient supply rates (PRS resin probes) and projected into the ordination diagram. To test for differences in soil nutrient supply rate and soil microbial community structure across forests and between declining and non-declining trees, we used Permutational Multivariate Analysis of Variance (PERMANOVA; McArdle and Anderson 2001; Anderson 2001). PERMANOVAs were performed applying the *adonis* function from the *vegan* package (Oksanen et al., 2013).

3. Results

3.1. Soil physical and chemical characteristics

No differences were found between declining and non-declining trees within each forest regarding soil hydraulic conductivity (K_s) (Fig. 1). Declining Scots pine forests showed extremely low values of saturated sorptivity (S) and this variable was lower in the two pine forests than in the Silver fir forest ($F = 5.22$, $p = 0.027$). We found no significant differences in soil nutrient content between declining and non-declining trees within each forest despite differences between forests were evident (Table 2; Supporting Information, Fig. S2).

Fig. 1. Differences observed in soil hydraulic conductivity (K_s) and sorptivity (S) across forest types and between declining (empty bars) and non-declining (solid bars) trees.

3.2. Soil nutrient supply rates

When the soil nutrient supply rates for the different elements were analyzed using NMDS, we found significant differences in soil nutrients between forest types (Fig. 2a), but no significant interaction between forest and tree vigour using PERMANOVA. Along these lines, we found significant differences between forests for the supply rates (estimated using PRS resin probes) of most of the mineral elements studied, but not between declining and non-declining trees (Table 2; Fig. 3). This was the case of NO₃, Ca, Mg, K, P, Fe, Al, Mn, Zn, Cu, S and Pb but not for NH₄, B and Cd. Thus, we found little support for the existence of differences in soil nutrient content and nutrient supply rates between declining and non-declining trees within each forest.

Table 2. Summary of the models and analyses of soil nutrient contents and soil nutrient supply rates estimated using resin probes. Models were fitted using Generalized Least Square models. A first model containing, forest, tree vigour (declining vs. non-declining trees) and their interaction was fitted. Multimodel selection based on information criteria was applied to select the best model. The model showing the lowest corrected Akaike Information Criteria (AICc) was selected as the best model. Some of the variables were log-transformed ($\log(x+1)$) to reduce skewness. For each variable, we show whether the variables were transformed (Y) or not (N), the most parsimonious model, $\Delta AICc$, and the model Akaike weight (W_i) or probability that the selected model is the best one, and coefficients associated to each variable and their significance (* $P < 0.05$; ** $P < 0.01$). The inclusion of forest in the final model is based on the existence of differences between forests.

Variable		Log-transformation	$\Delta AICc$	W_i	Forest type	Tree vigour	Forest type * tree vigour
Soil nutrient content	C	Y	3.05	0.82	9.16**		
	N	N	1.42	0.66	6.44**		
	C/N	N	3.59	0.86	71.71**		
Soil nutrient supply rate	NO ₃	Y	7.88	0.98	26.54**		
	NH ₄	Y	3.92	0.88			
	Ca	N	7.11	0.97	12.05**		
	Mg	N	7.72	0.97	7.37**		
	K	Y	5.89	0.92	6.49**		
	P	Y	7.22	0.97	7.02**		
	Fe	Y	9.16	0.99	13.41**		
	Mn	Y	9.00	0.99	12.1**		
	Cu	Y	6.82	0.97	11.65**		
	Zn	Y	7.74	0.97	8.89*		
	B	Y	2.15	0.62			
	S	Y	9.34	0.99	19.52**		

Pb	Y	7.25	0.96	7.04**
Al	Y	5.73	0.90	6.01*
Cd	Y	1.28	0.64	

3.3. Soil microbial community structure

We observed a significant interaction between forest and tree vigour in soil microbial community structure derived from PLFA analyses, indicating that there exist differences between declining and non-declining trees (PERMANOVA main effect of forest $F = 25.75$; $p < 0.01$; interaction between forest and status $F = 4.77$, $p < 0.01$ Fig. 2b). In the Silver fir and Aleppo pine forests, declining and non-declining trees showed marked differences in soil microbial community structure whereas no differences were found in the case of the Scots pine forest (Fig. 2b). We found a strong correlation between the first axis of the soil microorganism's ordination (NMDS axis 1) and the PRS resin probes estimated content of NO_3 ($r = 0.52$; $p < 0.01$), K ($r = 0.58$; $p < 0.01$) and S ($r = 0.50$; $p < 0.01$) in the soil, and a negative correlation with Fe ($r = -0.53$; $p < 0.01$), Cu ($r = -0.53$; $p < 0.01$) and Al ($r = -0.48$; $p = 0.01$) soil estimated content. The second axis (NMDS 2) was related with the concentration of NO_3 ($r = 0.49$; $p < 0.01$), P ($r = 0.53$; $p < 0.01$) and Cd ($r = 0.44$; $p = 0.02$) in the soil.

Table 3. Summary of the PLFA samples. Models were fitted using Generalized Least Square Models. A first model containing, forest, tree vigour (declining vs. non-declining) and their interaction was fitted. Multimodel selection based on AIC criteria was applied to select the best model. The model showing the lowest AICc was selected as the best model. Some of the variables were log-transformed ($\log(x+1)$) to reduce skewness. For each variable, we show the best fitted model, ΔAICc , W_i or model weight (probability that the selected model is the best one), and coefficients associated to each variable and their significance (* $p < 0.05$; **, $p < 0.01$). The inclusion of forest in the final model is assumes differences between forest soils, whereas the inclusion of the interaction between forest and tree vigour assumes differences in soil type between tree vigour classes within each forest.

	ΔAICc	W_i	Forest type	Tree vigour	Forest type * tree vigour
Total Biomass	5.25	0.93	23.56**	0.09	5.63*
Eukaryote	2.65	0.79	7.17**	0.61	4.45*
Gram Negative	8.83	0.99	21.22**	1.22	7.49**
Gram Positive	4.94	0.92	12.43**	0.47	5.73**

Actinomycetes	7.14	0.97	35.58**	0.31	7.00**
Fungi	3.92	0.88	20.71**		
AM Fungi	1.48	0.60	3.90*		

Table 4. Comparisons of the abundances of the major groups of soil microorganisms between declining and non-declining trees within each forest type. A separate two-way ANOVA was performed to compare the major groups of microorganisms in which we found a significant interaction between forest type and tree vigour (see Table 3). For each comparison, the F statistic and its associated probability level (p) are shown.

	Scots pine		Silver fir		Aleppo pine	
	F	p	F	p	F	p
Total Biomass	2.64	0.11	5.26	0.03	3.66	0.07
Eukaryote	0.03	0.67	6.66	0.02	3.36	0.08
Gram Negative bacteria	3.66	0.07	5.35	0.03	5.74	0.03
Gram Positive bacteria	3.25	0.08	5.37	0.03	3.54	0.07
Actinomycetes	1.99	0.20	5.01	0.03	5.06	0.04

Fig. 2. Non-Metric multidimensional scaling (NMDS) biplots of a) soil nutrient supply rates estimated using resin probes; b) and soil microbial composition based on PLFA (Phospholipid-derived fatty acids) for the three forest types. The correlation between PRS (Plant Root Simulator resin probes) soil nutrient composition and PLFA NMDS axes was projected in the ordination diagram. The polygons indicate the centroid of the distribution of the trees' groups. Declining and non-declining trees are indicated by open and filled circles, respectively, for the three studied forests.

When the major groups of soil microorganisms were analysed separately we found significant differences between declining and non-declining trees (Table 3, Fig. 4). In most cases, we found a significant interaction between forest and tree vigour which suggest differences between declining and non-declining trees (Table 3). The only exception to this rule were the groups of fungi and AM fungi (Table 3). When separate analyses were performed for each forest we found that the total biomass of microorganisms, eukaryotes, gram positive and negative bacteria, and actinobacteria differed significantly between declining and non-declining trees in the silver fir forest (Table 4). In the Aleppo pine forests only Gram negative bacteria and actinobacteria showed significant differences between classes of tree vigour. However, none of the analyzed microorganism types differed between declining and non-declining trees in the Scots pine forest (Table 4).

Fig. 3. Differences in soil nutrient supply rate (shown as $\mu\text{g}/10\text{cm}^2/91 \text{ days}$) across forest types and between declining (open boxes) and non-declining (solid boxes) trees.

Fig. 4. Univariate analysis of the PLFA (Phospholipid fatty acids) showing the relative abundance of major groups of soil microbes across the three forest types and between declining (open boxes) and non-declining trees (solid boxes). The total biomass of microbes is expressed in nanomoles per gram of dry soil; the abundance of the rest of the groups is expressed as a log-ratio of the proportion.

4. Discussion

We found evidence that drought-induced forest die-off is linked to changes in soil characteristics below coexisting trees of different vigour. Despite we found no differences in soil nutrient content and soil nutrient supply rates between declining and non-declining trees (Table 2, Fig. 2), we detected that soil microbial community structure differed significantly between declining and non-declining trees in two of the three forests studies (Tables 3 and 4, Figs. 2 and 4). Only in the Scots pine forest, where mortality rates peaked after the drought and die-off was more widespread than in the other sites (Table 1), we did not find any differences in soil microbial community structure between declining and non-declining trees (Table 4; Fig. 2). Importantly, differences between coexisting declining and non-declining trees are specific to each forest type and tree species. These results indicate that soil conditions might change because of tree decline across entire stands, and this suggests that differences between declining and non-declining trees are more prone to be detected in soil microbiota than in soil nutrients. Notably, we found that a high proportion of soil samples in two of the three studied forests, the Scots pine and the Aleppo pine forest, presented very low saturated sorptivity (S) values (Fig. 1). Since these S values were smaller than those reported in the literature for similar texture (Minasny and McBratney, 2000; Moret-Fernández et al., 2017) they could be related to a soil hydrophobicity phenomenon. Hydrophobicity could drastically reduce the affinity of soil for water after prolonged drought periods (Hallett et al., 2011). These results suggest that the studied forests diverge in their ability to process rainfall after prolonged droughts, which might exacerbate drought impacts on tree vigour.

Saturated sorptivity (S) was significantly lower in the Scots pine and Aleppo pine forests than in the Silver fir forest but no differences were found in hydraulic conductivity (K_s) between forests. The results obtained point to the existence of soil hydrophobicity or water repellence in the pine forests studied (Doerr et al., 2000, Hallett et al., 2011). Soil water

repellence have been found to be related with soil texture, dissolved organic compounds, dry periods and some other natural perturbations such as fire or drought (Hallett et al., 2011). Deciphering whether the observed soil water repellence is cause or consequence of the forest die off is out of the scope of this study, but it should be a research priority. Soil water repellence could exacerbate the effects of drought on tree health by reducing soil water infiltration following the first rainfall events after prolonged dry spells often characterized by high temperatures thus increasing drought stress and predisposing to forest die off (Goebel et al., 2011). However, the death of trees can also favour soil water repellency by changing the composition of soil organic contents and increasing light availability due to needles loss (Lebron et al., 2007). We found the lowest values of sorptivity in the Scots pine forest (Fig. 1) in which only 9 out of 38 trees were marked as dead trees in 2012 whereas in 2015 only 6 trees remained alive. In conifer forests, higher soil organic matter is often linked to increased soil water repellency during droughts as has been shown in Aleppo pine forests under semi-arid conditions (Mataix-Solera et al., 2007). In previous works, we attributed the strong Scots pine mortality observed in the region to the rise in air temperatures during early 2012 associated to climate warming but soil water repellence could be also a contribution factor to explain tree death (Camarero et al., 2015). Low values of sorptivity were also found in Aleppo pine forest studied, but this site is in a semi-arid region where both trees and soil microbes might be better adapted to low soil water contents, which characterizes some of these gypsum-rich substrates (Gazol et al., 2017). Furthermore, mortality rates were lower there than in the most affected Scots pine forest (Camarero et al., 2015). Overall, longer drought and warmer soil conditions enhance soil water repellency (Goebel et al., 2011), thus further research is required to investigate whether such low values of soil sorptivity and strong soil water repellency are cause or consequence of tree death.

We found no differences in soil nutrient concentrations and soil nutrients supply rates between declining and non-declining trees. This pattern can be explained by different reasons. Most of the nitrogen in the soil is contained in dead organic matter as insoluble polymers and some microorganisms are responsible of transforming it to dissolved organic nitrogen forms accessible to trees (van Der Heijden et al., 2008). However, trees and free-living soil microorganisms, particularly bacteria, can compete for the same sources of organic nitrogen (van der Heijden et al., 2008; Dannenmann et al., 2009). Thus, tree death interrupts nutrient uptake and carbon exudation by roots (Brunner et al., 2015), inducing in a short-term change in soil microbial composition that might consume the available nitrogen that trees are not using anymore. Similarly, and due to the short distance between the studied non-declining trees and other non-studied healthy trees, there is a chance that roots from neighbouring trees maintain the balance of soil nutrients in the surroundings of declining trees (Curiel Yuste et al., 2012). Nevertheless, the most plausible explanation is that more time is required to detect changes in soil nutrients after forest die-off or tree death (Dannenmann et al., 2009; Curiel Yuste et al., 2012). For example, organic matter from death trees can have large amounts of lignin and other compounds that need time to be decomposed (van Der Heijden et al., 2008; Pold et al., 2015; Baldrian 2017). In conclusion, differences between declining and non-declining trees in soil microbial community structure can be detected rapidly after tree death but more time might be required to detect differences in soil nutrient content.

We also found marked changes between declining and non-declining trees in two of the study sites, which highlight the strong specificity of the rhizosphere at fine spatial scales (Curiel Yuste et al., 2012; Koorem et al., 2014; Mikkelsen et al., 2016), despite soil microbiota also varies strongly from local to regional scales (Aponte et al., 2013; Lloret et al., 2015; Gazol et al., 2016; Tedersoo et al., 2016). Stursova et al. (2014) found drastic changes in soil microbial communities, particularly in fungi species, after a rapid bark beetle-induced

tree dieback. They attributed most of the changes in the soil microbiota to the drastic increase of soil organic matter amount as a consequence of the rapid fall of dead needles and branches. However, in our study, we were comparing declining and non-declining trees at small spatial scales (a few meters) and thus we do not expect major changes in canopy-derived organic matter. In contrast, rhizodeposition rates can be shifted by droughts (Preece and Peñuelas 2016). Along these lines, Curiel Yuste et al. (2012) found that soils beneath dead, declining and non-declining Scots pine trees differed in soil bacterial community composition while no major differences in soil nutrient contents were found. These results highlight that soil microbial communities are able to respond rapidly to forest die-off and tree decline induced by severe droughts (Cai et al., 2010; Lloret et al., 2015), *although more time might be required to detect changes in soil nutrient content.*

Despite strong differences in soil microbial community structure between declining and non-declining trees in the Aleppo pine and Silver fir forests, we found no differences at all in the Scots pine forest where declining trees presented the lowest values of saturated sorptivity (Table 4, Figs. 1 and 2). We hypothesize that this particular behaviour is due to the strong mortality observed in the Scots pine forest after 2012 (76% of trees tagged in 2012 were dead in 2015). While in the Aleppo pine and Silver fir forests tree decline, and die-back is spatially heterogeneous, in the Scots pine forest the die-off event was widespread and some of the individuals classified in 2012 as non-declining trees show evident signs of decline or have died three years later.

5. Conclusions

The links between drought, soil nutrient availability, shifts in the soil microbiota and forest die-off are still little understood. Here we provide relevant information to start filling some of these research gaps. The composition of soil microbiota seems to be a more rapid proxy and

monitor of forest die-off changes than soil nutrient contents and availability. We found no differences in soil nutrient content between trees of different vigour, which contrasts with other regional studies in mesic forests indicating that nitrogen-poor soils were associated to tree decline (Pinto and Peñuelas 2007). This discrepancy may be caused by the overwhelming role played by drought as trigger of die-off in the study cases leading to a shift in the microbiota inhabiting the soil below declining or recently dead trees. Lastly, the link between soil hydrophobicity and tree death should be further explored to decipher whether it is a cause or a consequence of forest die-off in sight of forecasted more frequent and intense drought events. Measuring or modifying soil microbial community structure and soil-water retention capacity can be useful monitoring or management tools to anticipate and buffer the impacts of drought-induced forest die-off.

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703

Graphical Abstract

Differences in soil microbial community structure of declining (open symbols) and non-declining (filled symbols) trees in three forest types subjected to contrasting climatic conditions (Silver fir, black symbols; Scots pine, green symbols; Aleppo pine, red symbols). The graph shows the Non-Metric multidimensional scaling (NMDS) biplot of soil microbial composition based on PLFA (Phospholipid-derived fatty acids). The correlation between soil nutrient composition and NMDS axes was projected in the ordination diagram. The polygons show the centroid of the distribution of the trees' groups. The two trees with different vigour correspond to the Silver fir forest.

Supporting Information for the manuscript “Beneath the canopy: linking drought-induced forest die off and changes in soil properties” by Gazol et al.

Supporting information captions

Table S1. Soil texture in the three studied forests

Figure S1. August 12-months long Standardised Precipitation–Evapotranspiration Index (SPEI).

Figure S2. Soil, Carbon (C), Nitrogen (N) and C/N ratio values observed in the three studied forests.

Table S1. Soil texture of the three studied forests.

Site – Tree species	Sand (%)	Lime (%)	Clay (%)
Paco Ezpela - Silver fir (<i>Abies alba</i>)	45.58	41.20	13.22
Corbalán - Scots pine (<i>Pinus sylvestris</i>)	76.49	21.13	2.38
Peñaflor - Aleppo pine (<i>Pinus halepensis</i>)	71.18	23.76	5.05

Figure S1. August 12-months long Standardised Precipitation–Evapotranspiration Index (SPEI; Vicente-Serrano et al. 2010). The SPEI is a climatic proxy widely used for drought quantification (see Vicente-Serrano et al. 2010). Positive and negative values indicate wet and dry conditions, respectively. The red arrows indicate the SPEI values observed in 2012. Different colours and symbols are used for each site: Corbalán (*P. sylvestris* stand; green lines and squares); Paco Ezpela (*A. alba* stand; black lines and circles); Peñaflor (*P. halepensis*; red lines and triangles).

Figure S2. Differences in soil carbon, nitrogen and C/N ratio across forest types and between declining (open boxes) and non-declining (solid boxes) trees. The C and N values represent the percentage of the soil elements in the sample.

Highlights

- Drought-induced forest die-off may impact soil nutrients and microbiota.
- Die-off alters soil microbial community structure substantially.
- Soil nutrient availability does not change as a function of tree vigor.
- Soil water repellence amplifies drought stress.
- The composition of soil microbiota is a monitor of forest die-off impacts.