Effects of past and current drought on the composition and diversity of soil microbial communities

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Abstract

Drought is well known to have strong effects on the composition and activity of soil microbial communities, and may be determined by drought history and drought duration, but the characterisation and prediction of these effects remains challenging. This is because soil microbial communities that have previously been exposed to drought may change less in response to subsequent drought events, due to the selection of drought-resistant taxa. We set up a 10-level drought experiment to test the effect of water stress on the composition and diversity of soil bacterial and fungal communities. We also investigated the effect of a previous long-term drought on communities in soils with different historical precipitation regimes. Saplings of the holm oak, *Quercus ilex* L., were included to assess the impact of plant presence on the effects of the drought treatment. The composition and diversity of the soil microbial communities were analysed using DNA amplicon sequencing of bacterial and fungal markers and the measurement of phospholipid fatty acids.

The experimental drought affected the bacterial community much more than the fungal community, decreasing alpha diversity and proportion of total biomass, whereas fungal diversity tended to increase. The experimental drought altered the relative abundances of specific taxa of both bacteria and fungi, and in many cases these effects were modified by the presence of the plant and soil origin. Soils with a history of drought had higher overall bacterial alpha diversity at the end of the experimental drought, presumably because of adaptation of the bacterial community to drought conditions. However, some bacterial taxa (e.g. *Chloroflexi*) and fungal functional groups (plant pathogens and saprotrophic yeasts) decreased in abundance more in the pre-droughted soils.

Our results suggest that soil communities will not necessarily be able to maintain the same functions during more extreme or more frequent future droughts, when functions are

43 influenced by community composition. Drought is likely to continue to affect community
44 composition, even in soils that are acclimated to it, tending to increase the proportion of fungi
45 and reduce the proportion and diversity of bacteria.

46

47 **1. Introduction**

48 Drought is a serious problem in many parts of the world, and the impacts on plants, in both
49 natural and agricultural settings, are increasingly well documented. The effects of water stress
50 on soils and their associated biota, however, remain less certain, even though water stress
51 may be the most frequent environmental stress experienced by soil microorganisms (Schimel
52 et al., 2007), with demonstrated impacts on soil properties and microbial communities.
53 Severe and prolonged water stress, in the most extreme scenarios, leads to desertification but
54 may have important consequences for soil health much sooner. An estimated one fifth of the
55 Earth's soil is currently acutely degraded and showing declining productivity, often due to
56 drought-related phenomena (United Nations Convention to Combat Desertification, 2017).
57 Maintaining healthy soils is important because soils are fundamental for a wide range of
58 ecosystem services, including food security, nutrient cycling, timber production, and climatic
59 regulation. Moreover, the increasing research into belowground processes and plant-soil
60 interactions and the availability of methods for studying microbial communities have raised
61 interesting fundamental questions about the role of roots in mediating the effect of drought on
62 soil microbial communities.

63 Drought can directly affect microbes by desiccation or resource limitation, because
64 substrate diffusion is reduced at low levels of soil moisture (Schimel et al., 2007; Naylor and
65 Coleman-Derr, 2017). Drought experiments have reported decreases in microbial biomass
66 and activity (Hueso et al., 2012; Alster et al., 2013; Hartmann et al., 2017; Castaño et al.,

2018), reductions in carbon and nitrogen mineralisation (Hueso et al., 2012), and accumulation of solutes, such as amino acids (in bacteria) and polyols (in fungi), which help prevent dehydration but are energetically expensive (Schimel et al., 2007). Drought may also have indirect effects, through interactions with plants, because plants can have species-specific effects on rhizosphere microbiota mediated by rhizodeposits (Bergsma-Vlami et al., 2005; Haichar et al., 2008; Bressan et al., 2009; Ladygina and Hedlund, 2010; Philippot et al., 2013; Lareen et al., 2016). Plants may have a protective effect on microbes that live in or near the rhizosphere, at least when normal root function can be maintained. The tolerance of a plant species to drought can therefore be important for the soil community in the immediate vicinity since the presence of a tolerant plant may modulate the impacts on soil.

Soil microbial communities vary greatly at all geographical scales, depending on factors such as the chemical properties of the soil, the climate, and the plant community, but some general factors associated with drought have been identified. For example, evidence suggests that fungi are more tolerant than bacteria to water stress (Bapiri et al., 2010; Barnard et al., 2015; de Vries et al., 2018), and Gram-positive bacterial lineages are generally more drought resistant than Gram-negative lineages, perhaps due to their thicker cell walls (Schimel et al., 2007). Some evidence suggests that among fungi, yeasts may have a high tolerance to drought, because they tend to be more common in more extreme environments and tend to reproduce by budding, which is generally a more stress tolerant strategy of reproduction (Treseder and Lennon, 2015). Water stress may affect the taxonomic diversity of microbial communities, and both decreases (Bouskill et al., 2013) and increases (Acosta-Martínez et al., 2014) have been reported. Microbial communities with high diversity, and particularly high functional diversity, may be more tolerant to drought (and to other perturbations), but this tolerance is likely to be strongly associated with a range of biotic and

abiotic features of the soil (Griffiths and Philippot, 2013). Many uncertainties about the response of microbial communities to water stress, though, remain.

Both soil microbial communities and droughts are highly variable, in both natural and experimental environments. The impact of water stress on plants and soils can depend on the timing or duration of the event or treatment (Hoover and Rogers, 2016; Mengtian et al., 2018), the proportional change in water availability, and the historical precipitation regime (Evans and Wallenstein, 2012; Bouskill et al., 2013). On this last point, soils that have experienced drought events may demonstrate 'legacy effects' whereby the soil community continues to show the impact of the drought for many years, and it may even modify the response to a later drought (de Vries et al., 2012; Bouskill et al., 2013; Kaisermann et al., 2017; Meisner et al., 2018). The impact of drought may also not elicit a linear response (Knapp et al., 2017), meaning that any negative effect may decrease or increase as the drought progresses, indicating acclimation or a 'tipping point', respectively. All this variability adds to the difficulty of drawing conclusions about the impacts of drought on soil microbes, and experiments are needed to test a more complex range of drought scenarios, including multiple levels of drought intensity in space and time and investigating if and when biologically relevant thresholds are exceeded (Beier et al., 2012).

The main objective of this study was to investigate the effect of increasing drought intensity on the bacterial and fungal communities of a Mediterranean soil. We focused on a holm oak (*Quercus ilex* L.) forest system, which is a predominant habitat throughout the Mediterranean Basin where greater drought frequency and severity is predicted (Field et al., 2014; Touma et al., 2015). We set up a greenhouse experiment with ten levels of drought and then used DNA-based amplicon sequencing and lipid analyses to observe the effects on soil bacterial and fungal communities in pots containing *Q. ilex* saplings. This experiment allowed us to determine whether bacterial communities were more responsive than fungal

communities to drought and if we could identify changes in functional groups. We also wanted to determine if the impacts of drought were mitigated or increased by the presence of the plants and by the historical precipitation regime of the soil, and to determine the effect of drought intensity. We hypothesised that: (1) drought would affect microbial community composition, negatively affect diversity, and affect bacteria more than fungi, (2) the presence of *Q. ilex* would decrease the impacts on the microbial communities, and (3) soils with a history of drought would be more resistant to the drought treatment.

2. Materials and methods

2.1. Plant and soil material

A greenhouse experiment was established in May 2015 at the experimental fields of the Autonomous University of Barcelona (Spain). The experiment comprised 180 pots of 3.5 l, half of which (90 pots) were planted with three-year-old *Q. ilex* saplings (provided by Forestal Catalana, Barcelona, Spain). The other 90 pots contained substrate only. The substrate used in all pots consisted of 45% autoclaved peat (121ºC for 60 mins), 45% sand, and 10% natural soil inoculum. Soil was collected from a south-facing slope (25%) in a natural holm oak forest in the Prades Mountains in northeastern Spain (41°13′N, 0°55′E; 930 m a.s.l.). This forest is the site of a long-term drought experiment that began in 1999 and reduces precipitation throughfall by approximately 30% (Ogaya and Peñuelas, 2007). There were three soil inocula, each with 60 replicates, which had different prior treatments: control, pre-droughted, or pre-sterilised. We collected topsoil from the treatment plots of the long-term drought experiment. Soil from the control plots was used as the inoculum for the corresponding control soil in our experiment and was autoclaved (121ºC for 60 mins) for use in our pre-sterilised treatment, and soil from the drought plots was used as the inoculum in

our pre-droughted treatment. This pre-droughted soil allowed us to test for 'legacy' effects of the long-term drought. The pre-sterilised treatment was incorporated in order to try to separate the effects of the previous soil community from the drought and plant effects and remove any potential idiosyncratic features of the historical soil community. The roots of the *Q. ilex* saplings were carefully washed in tap water before transplantation to remove soil from the previous potting mix, so that the soil communities were representative of the three new soil treatments. Whilst complete removal of previous substrate and original rhizosphere microbes was not possible without causing damage to the roots, the soil microbes in the bulk soil would have been predominantly composed of those from the new inocula. All plants were then allowed to adjust to the greenhouse environment for six weeks, receiving daily watering (until the end of June 2015), after which they were top-watered every day with amounts sufficient to maintain soil moisture at 20-25%.

2.2. Experimental design

The drought treatment consisted of ten levels of drought, applied by withholding water for 0, 2, 4, 7, 9, 11, 14, 16, 18, and 21 days. Each drought level therefore had 18 pots, divided into six replicate blocks (Supplementary Material Fig. S1). Soil samples were collected at the end of the drought period specific for each drought level. For pots with zero days of drought this sampling occurred at the end of the six week acclimation period. A subset of 90 of these soil samples were used for the DNA sequencing analysis, representing all drought levels and all soil history and plant treatments. This included 13-15 replicates for each combination of soil history × presence of *Q. ilex*. One sample was discarded for the bacteria and two for the fungi (see Supplementary Material, Table S1 for full details of replication). Air temperature was monitored throughout the experiment using an EL-USB-2 data logger (Lascar Electronics, Wiltshire, UK) and had a mean of 26.7 °C. Soil temperature averaged 27.0 °C across the

three soil types (Decagon Em50 data logger with 5TM soil probes, Decagon Devices, Pullman, USA) (Supplementary Material Fig. S2). Soil moisture in each pot was measured at the start of the experiment and at the end of its drought period using an ML3 Theta Probe connected to a HH2 Moisture Meter (Delta-T Devices, Cambridge, UK). Mean soil moisture was 22.6% at the start of the experiment and decreased exponentially to 0.3% by the end of the 21-day drought treatment, and this did not differ significantly between pots with plants and those without (Supplementary Material Fig. S3).

2.3. DNA library preparation and sequencing

Total community DNA was extracted from approximately 0.25 g of soil using a PowerSoil DNA Isolation Kit following the manufacturer's protocol (MoBio, Carlsbad, USA). The hypervariable V3-V4 regions of the bacterial 16S rRNA gene was amplified using the 341F – 806R primer pair (Klindworth et al., 2013) modified to include Illumina adapter sequences. Each 25 µl reaction mixture contained 1.5 µl of undiluted DNA extract, 1 µl each of the forward and reverse primers (10 µM), and 12.5 µl of Phusion High Fidelity PCR Master Mix with HF Buffer (ThermoFisher Scientific, Waltham, USA). Initial DNA concentration ranged between 3.9-10.2 ng/µl and DNA concentration was not standardized prior to PCR. PCR conditions were: initial denaturation at 98 °C for 30 s, followed by 30 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 4 min. After confirming successful amplification by agarose gel electrophoresis, PCR products were purified and normalized using Sequalprep plates (Thermofisher, USA), and subject to a second indexing PCR such that each sample received a unique combination of 6-nucleotide barcoded forward and reverse primers. The reaction mixture was as above, and the PCR program was an initial step at 95°C for 30 s, 8 cycles of: 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; and a final step of 72°C for 5 min. PCR products

were again purified and normalized with Sequalprep plates and pooled for sequencing. The fungal ITS1 region was amplified in a one step approach using the primers ITS1f and ITS2 augmented with multiplexing barcodes (Smith and Peay, 2014). Each reaction mixture contained 1 µl of the DNA extract, 1 µl of forward and reverse primers (10 µM), 200 µM dNTP's, 1X GC buffer and 0.4 U of Phusion DNA polymerase. PCR conditions were: initial denaturation at 98 °C for 30 s, followed by 40 cycles of 98 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. Samples that failed to produce a PCR product were discarded, and PCR was repeated. Some fungal samples still failed to produce usable PCR products and were excluded from further analyses, but this only represented three samples out of 88 in total. Fungal PCR products were also purified and normalized with Sequalprep plates, and additionally extracted from a 1.5 agarose gel for size selection (approximately 200-500 bp which covers the entire range of length variation in the fungal ITS1 region) and to remove primer dimers. Then they were additionally purified using a QIAquick Gel Extraction Kit (Qiagen, Venlo, The Netherlands). Both fungal and bacterial libraries were quantified with real-time PCR using KAPA Library Quantification Kits (Kapa Biosystems, Wilmington, USA) to determine dilution factors for the sequencing protocol.

The libraries were sequenced on the Illumina MiSeq platform (Illumina Inc., San Diego, USA), with 2 x 300 cycles (V3 chemistry) for forward and reverse reads for bacteria, and 300 cycles (V2 chemistry) in the forward direction only for fungi. The reproducibility of sample preparation and sequencing was tested by sequencing a small number of technical replicates (DNA isolated from the same samples but subjected to independent PCR reactions with distinct primer barcodes).

2.4. Quality filtering and bioinformatic analysis

The initial bioinformatic analysis of bacterial sequences used USEARCH software (Edgar, 2013). Merging of paired-end reads was attempted, but low quality basecalls in the tail of both read directions precluded satisfactory merging. We therefore proceeded with separate parallel analyses of the forward and reverse reads (after truncating to 180bp and 150 bp respectively). Both reads gave qualitatively very similar results, so we focus hereafter on the longer forward read data. After primer removal sequences were filtered by quality leaving a total of 2.4 M high-quality sequences. Replicate and singleton sequences were removed, and a set of representative sequences of operational taxonomic units (OTUs) (97% similarity) was constructed using the UPARSE-OTU algorithm (Edgar, 2013). Chimeras were removed (leaving 11890 non-chimeric OTUs), and all original reads were mapped to the non-chimeric OTUs using the USEARCH algorithm with global alignments at an identity threshold of 0.97, yielding an OTU table. All subsequent steps used QIIME (Caporaso et al., 2010b). OTUs were aligned using the PyNAST algorithm (Caporaso et al., 2010a) and the Green Genes database (release 13_8, DeSantis et al., 2006) as a template alignment. A subsampled OTU table was created by randomly sampling the original OTU table to avoid artefacts associated with library size. Samples that contained fewer sequences than the requested depth (2480) were omitted from the output OTU tables. Each OTU was taxonomically identified based on the 97% Green Genes database (release 13_8) using the RDP classifier (Wang et al., 2007).

Fungal sequences were analysed using USEARCH following the UPARSE pipeline (Edgar, 2013). The sequences were trimmed to 250 bp and filtered for quality (maximum expected error of 0.5), leaving a total of 9.79M sequences. While we only sequenced the forward reads, in some instances (sequences shorter than 250 bp) the reverse primer is found at the end of the forward reads and these were removed. Then, Ns were added up to 250 bp for efficient clustering of the OTUs. Singleton sequences were removed, and all others were

clustered to 97% similarity. Chimeras were filtered de novo and through the UNITE database of ITS1 sequences implemented in UCHIME, leaving a total of 3,323 non-chimeric OTUs, after which original sequences were mapped against these OTUs at a similarity threshold of 97% and assembled in an OTU table. Representative sequences for each OTU were aligned to all fungal representative species in the UNITE database (Kõljalg et al., 2005) (release date 20.11.2016) using the BLAST algorithm with default settings. The resulting hits were assigned to taxa, selecting the hit with the lowest E-value, provided it had a minimum E-value of $1x10^{-36}$ and a minimum alignment length of 75 bp. OTUs were subsequently assigned to functional groups if a genus was provided for the highest hit, and if it matched one of the genera with known lifestyles provided by Tedersoo et al. (2014). When the genus level was unknown, lifestyle was assigned at the family level if >80% of the genera within that family (represented by more than three genera) had the same lifestyle. As for bacteria, the OTU table was downsampled, to 12,332 reads per sample.

2.5. Analysis of phospholipid fatty acids

We used the amounts of phospholipid fatty acids (PLFAs) to quantify microbial biomass and the ratio of fungal to bacterial biomass. PLFA extraction and identification followed Frostegård et al. (1993), using 1 g of freeze-dried soil from each replicate. The abundance of individual fatty acids was determined as nmol per g of dry soil, and standard nomenclature was used (Tunlid et al., 1989). Concentrations of each PLFA were calculated based on the 19:0 internal standard concentrations. Selection of bacterial PLFAs follows the selection of fatty acids by Frostegård and Bååth (1996), of which i14:0, i15:0, a15:0, i16:0, 16:1ω7c, a17:0, i17:0, cy17:0, 18:1ω7, and cy19:0 were present in our samples. We calculated the sum of i14:0, i15:0, a15:0, i16:0, a17:0, and i17:0 as an indicator of Gram-positive bacteria. Gram-negative bacteria were identified by the PLFAs 16:1ω7c, cy17:0, 18:1ω7, and cy19:0

(Zelles, 1999). The fungi were identified by 18:2ω6 (Frostegård et al., 1993; Frostegård et al., 2011). The ratio of 18:2ω6 to total bacterial PLFAs was used to estimate the ratio of fungal to bacterial biomass in soils (Bardgett et al., 1996; Frostegård and Bååth, 1996).

2.6. Statistical analyses

All statistical analyses were carried out using R v3.4.1 (R Core Team, 2016). The data for the fungal and bacterial communities were log-transformed relative abundance of each OTU within a sample. Non-metric multidimensional scaling (NMDS) plots (using Bray-Curtis distance with the *metaMDS* function in *vegan*) were constructed to visualize the separation between treatments. A permutational multivariate analysis of variance (PERMANOVA) was used to assess the effects of drought, soil history, and presence of a plant on the microbial communities, using the *adonis2* function in the *vegan* package. Block was included as a random factor, and 3000 permutations were used. This used Bray-Curtis dissimilarity indices generated by the *vegdist* function (also from *vegan*). A pair-wise PERMANOVA analysis was used to test for significant differences between pairs of factor levels (Martinez Arbizu, 2017). We also carried out an NMDS analysis and PERMANOVA with the pre-sterilised soil removed, to check that this sterilised soil was not driving all of the significant soil history effect, and of the non-droughted pots only to assess the differences in the soil communities under ambient water conditions.

Shannon diversity (H) and Simpson index were calculated as a measure of community alpha diversity (later referred to simply as community diversity) using the *diversity* function (in *vegan*) and analysed using linear mixed effects models (*lme* function in the *nlme* package) with drought intensity, soil history and plant-presence as predictor variables, and block as a random factor. The change in community diversity following drought (ΔH) was calculated for each soil and plant treatment as $(H_{dmax} - H_{con})/H_{con} * 100$, where H_{con} is the mean Shannon

diversity under normal water conditions (zero days of drought), and H_{dmax} is the mean Shannon diversity under the most extreme drought level (21 days of drought). Unfortunately, due to a lack of paired samples for H_{con} and H_{dmax}, only means per treatment combination could be calculated, and not standard errors. Also, a two sample *t*-test was done to compare the mean Shannon diversity of the bacteria and fungi communities under control conditions compared with the most extreme drought.

The relative abundance of bacterial phyla and classes with more than 2% mean relative abundance, and fungal functional groups with more than 1.5% mean relative abundance was analysed using linear regression, with drought intensity, soil history and plant-presence as predictor variables. PLFA data indicating total microbial abundance was analysed using a linear mixed-effects model (*lme* function in the *nlme* package) with block as a random factor. The fungal:bacterial ratio and the ratio of Gram-positive to Gram-negative bacteria were analysed with a generalised linear model with a quasi-binomial distribution.

In all analyses except relative abundance of taxa, drought was analysed both as a continuous and a categorical (non-ordinal) variable with levels grouped as control (0 days of drought), low-level drought (2-7 days of drought), mid-level drought (9-14 days of drought), and high-level drought (16-21 days of drought). These groups were selected in order to equally divide the drought treatments, each spanning a 6-day period. Results are usually shown with drought as a continuous variable, unless otherwise stated, in which case it is due to a non-linear relationship between the dependent variable and drought, specifically for Shannon diversity for fungi. In all cases, the *P*-values shown are the result of an ANOVA (*Anova* function in the *car* package) with type III sums of squares.

3. Results

3.1. Initial differences in the soil communities

The NMDS analysis suggested that soil history and the presence of the plant affected the composition of the bacterial community before the start of the drought treatment (after six weeks of acclimation) (Fig. 1a, Fig. 1b, Table 1; PERMANOVA, soil history effect, $P < 0.001$, pseudo $R^2 = 0.25$, plant effect, $P < 0.001$, pseudo $R^2 = 0.16$). Note that the soil effect was driven by the strong difference between the pre-sterilised soil and the control and pre-droughted soils (Pairwise PERMANOVA, $P < 0.05$), with no significant difference between the latter two soils. The presence of *Q. ilex* did not affect the fungal community (Fig. 1c), but soil history did, with the pre-sterilised soil clearly separated from the control and pre-droughted soils (Fig. 1d; PERMANOVA, soil history effect, $P < 0.001$, pseudo $R^2 = 0.51$).

The diversity of the bacterial community measured by the Shannon index was affected by an interaction between the soil history effect and the presence of the plant (Table 1; significant interaction, $\chi^2 = 29.5$, $P < 0.001$) such that the pre-sterilised soil had lower diversity than the other soils, but only in the absence of the plant (Supplementary Material, Fig. S4a). The Simpson index showed a very similar pattern, again with a significant interaction between plant and soil history (Table 1; significant interaction, $\chi^2 = 21.9$, $P < 0.001$), again with the lowest value for pre-sterilised soil without the plant (Supplementary Material, Fig. S5a).

Soil history, but not the plant, affected the diversity of the fungal community. Diversity of the fungal community was higher in the pre-droughted than the pre-sterilised soil (Table 1; $\chi^2 = 11.9$, $P < 0.01$), with the control soil intermediate, and the diversity was 14.5% higher in the pre-droughted than the control soil, but not significantly higher (Supplementary Material, Fig. S4b). The Simpson index showed a similar pattern (Supplementary Material,

339 Fig. S5b) although with an interaction between soil history and plant (Table 1; χ^2 = 9.3, P <
340 0.01), so that the pre-droughted soil without *Q. ilex* had lower diversity than the control soil
341 with *Q. ilex* present.

342
343 **3.2. Microbial abundance**

344 The fungal:bacterial ratio increased with experimental drought duration (χ^2 = 10.1, P < 0.01)
345 and was also higher in pots containing only soil than those containing *Q. ilex* (χ^2 = 34.2, P <
346 0.001) (Fig. 2a). Soil history had no effect on the fungal:bacterial ratio. Drought had no effect
347 on total microbial biomass, but soil history significantly interacted with the presence of *Q.*
348 *ilex* in the pots containing only soil (χ^2 = 24.3, P < 0.001). In the absence of *Q. ilex*, microbial
349 biomass was higher in the control and pre-droughted soils than the pre-sterilised soil.
350 However, microbial biomass was lower in the pre-droughted than the control soils when *Q.*
351 *ilex* was present, with intermediate biomass in the pre-sterilised soil (Fig. 2b). The ratio of
352 Gram-positive:Gram-negative bacteria decreased as drought duration increased (χ^2 = 5.5, P <
353 0.05) and was affected by soil history (χ^2 = 21.3, P < 0.001), being lower in the pre-sterilised
354 soil and in soils without *Q.* ilex (χ^2 = 11.5, P < 0.001) (Supplementary Material Fig. S6).
355

356 **3.3. Composition of the microbial community during the drought experiment**

357 Drought had a strong impact on the bacterial community (P < 0.001) but not the fungal
358 community (Table 2, Fig. 3). The result was almost identical when drought was grouped into
359 the four categories (control and low-, mid-, and high-level drought, Table 2), except for an
360 interaction between drought and the presence of *Q. ilex* for the bacterial community (Table 2;
361 P < 0.05). Although, note that the analysis without the pre-sterilised soil revealed an effect of
362 drought (P < 0.05) on the fungal community when drought was considered as a categorical
363 variable (Supplementary Material, Table S2). Differences between each pair of categorical

drought levels are shown in Supplementary Material (Table S3). Soil history was a significant driver of both the bacterial and fungal communities ($P < 0.001$), and there were significant differences (Pairwise PERMANOVA, $P < 0.01$) between all pairs of the three groups of soil history (Table S4). This significant effect of soil history was maintained even when the pre-sterilised soil treatment was removed from the analysis (Supplementary Material, Table S2), however note that the R^2 value decreases from 0.17 to 0.05, suggesting that the pre-sterilised soil drives much of this effect. The presence of *Q. ilex* was important for bacteria ($P < 0.001$) and fungi ($P < 0.001$). Soil history also influenced the effect of plant presence (there was a significant interaction) on both bacteria and fungi community composition (Table 2, Fig. 3), although for fungi this interaction was not seen when the pre-sterilised soil was removed from the analysis (Supplementary Material, Table S2).

3.4. Diversity of the microbial community during the drought experiment

Drought duration had a negative effect on the diversity of the bacterial community (Fig. 4a, Fig. S7a; Shannon diversity, $\chi^2 = 9.0$, $P < 0.01$, Simpson index, $\chi^2 = 6.2$, $P < 0.05$) but with no interaction with either soil history or the presence of *Q. ilex* (Table 3). The three soils also had different diversities, in the order pre-droughted > control > pre-sterilised (Shannon diversity, $\chi^2 = 12.7$, $P < 0.01$), and the effect of soil history was modified by the presence of a plant, with a strong plant effect increasing diversity in the pre-sterilised soil (Table 3, Fig.4b, Fig. S7b; Shannon diversity, $\chi^2 = 37.6$, $P < 0.001$, Simpson index, $\chi^2 = 19.4$, $P < 0.001$).

Drought had a positive effect on fungal Shannon diversity as a continuous variable ($\chi^2 = 3.9$, $P < 0.05$), but this effect was even greater when drought was separated into discrete groups (control and low-, mid-, and high-level droughts) ($\chi^2 = 21.6$, $P < 0.001$), with the low- and

high-level groups having higher Shannon diversities than the control group (Fig. 5a). This same pattern was shown for Simpson index (Table 3, Fig. S8a; $\chi^2 = 19.0$, $P < 0.001$). Drought duration did not interact with either the presence of *Q. ilex* or soil history for fungi (Table 3). Fungal diversity was generally lower in the pre-sterilised soil than the control and pre-droughted soils (Shannon diversity, $\chi^2 = 15.4$, $P < 0.01$), and soil history interacted with the presence of *Q. ilex*, with a larger positive effect of *Q. ilex* in the pre-sterilised soil (Fig. 5b, Fig. S8b; Shannon diversity, $\chi^2 = 9.0$, $P < 0.05$, and Simpson index, $\chi^2 = 13.6$, $P < 0.01$).

For bacterial communities, all combinations of soil history and plant-presence showed a decline in Shannon diversity between the control (0 days drought) treatment and the most extreme (21 days) drought treatment (a negative ΔH). Overall, for bacteria, a *t*-test on H in the control and the extreme drought treatments showed that the difference between Shannon diversity in the control and most extreme drought treatment was not significantly different (Table 4; $t = 1.72$, $P = 0.10$). In contrast, fungal Shannon diversity tended to increase between the most extreme drought treatment and the control drought level (a positive ΔH), by up to 35% in one case, although in the case of soil with a history of previous drought and with presence of *Q. ilex* Shannon diversity decreased slightly. Overall, for fungi, a *t*-test did not reveal a significant difference between the two groups (control – 0 days drought – and most extreme drought – 21 days drought) ($t = -1.88$, *P*-value = 0.07).

3.5. Microbial taxonomic composition

Overall, the most abundant bacterial phyla were *Proteobacteria* (36.0% of amplicon reads), *Actinobacteria* (18.5%), *Bacteroidetes* (13.4%), and *Verrucomicrobia* (6.4%). Other phyla that comprised a substantial (>2%) amount of the bacterial community were *Planctomycetes* (5.0%), *Chloroflexi* (4.8%), *Acidobacteria* (4.5%), and *Firmicutes* (2.9%). Drought affected

most of these phyla (Table S5), and there were often interactions with either the plant-presence or soil history. *Actinobacteria* and *Planctomycetes* abundance increased with drought whereas *Proteobacteria* abundance was negatively correlated with drought duration. For *Bacteroidetes* there was a negative correlation with drought in control soil, and for *Chloroflexi* abundance increased with drought in control soil but decreased with drought in pre-droughted soil.

Twelve bacterial classes were present at >2%: *Alphaproteobacteria* (17.5%), *Actinobacteria* (11.4%), *Betaproteobacteria* (6.9%), *Gammaproteobacteria* (6.4%), *Saprospirae* (5.6%), *Thermoleophilia* (5.5%), *Cytophagia* (5.3%), *Deltaproteobacteria* (5.1%), *Planctomycetia* (3.3%), *Opitutae* (3.0%), *Bacilli* (2.8%), and *Anaerolineae* (2.6%). Again, the drought treatment affected most classes, but the effect was highly variable and depended on the presence of *Q. ilex* and soil history (Table S6). A large proportion of the fungal OTUs (45.8%) were unidentified, but filamentous saprotrophic fungi were the most abundant functional group (48.3%), followed by plant pathogens (2.5%) and saprotrophic yeasts (1.9%) (Table S7). Other functional groups at lower proportions (< 1.5%) of the total abundance included mycoparasites, ectomycorrhizal fungi, and saprotrophic white-rot fungi.

4. Discussion

The impact of various drought scenarios on soil and rhizosphere microbes remains uncertain and is likely to depend on soil and plant properties. Few previous studies have measured the response of soil microbial communities to more than two levels of drought, a knowledge gap, which our study aimed to address. The experimental design allowed us to determine the effect of drought on microbial communities in detail, both as a continuous variable, to observe general trends, and grouping the drought levels, to increase replication in separate

groups and allow comparisons between drought intensities. Our results have demonstrated that bacteria and fungi can have complex responses to water stress that vary with the intensity of the drought as well as soil history and presence or absence of plants.

4.1. Differences in the soil communities without drought

Our analysis of pots in the zero days of drought (control) treatment, which were sampled after six weeks of acclimation in the greenhouse, revealed that *Quercus ilex* plants impacted bacterial community composition and had a positive effect on bacterial diversity but did not affect the composition or diversity of the fungal community. This result may indicate either a larger influence of the rhizosphere (e.g. root exudation) on bacteria than fungi, or that fungi community composition responds more slowly due to their longer generation times (Rousk and Bååth, 2011). Consistent with this supposed relative inertia of fungal communities, previous soil history was found to have had a strong influence on the fungal community, which may have masked any effect of the plant. Indeed, soil history clearly separated both the bacterial and fungal communities in the sterilised soil from those in the control and pre-droughted soils. This showed that the sterilisation treatment successfully removed most of the bacteria and fungi present in the soil, and that the new microbial communities that colonised the soil were different than the original ones. Soil history also affected the diversity of bacteria and fungi in pots with zero days of drought. Bacterial community diversity was higher in pre-droughted and control soils compared with sterilised soil, but only in the absence of *Q. ilex*, indicating that the presence of a plant can quickly restore bacterial diversity. This is supported by previous studies that have shown the importance of plants for increasing bacterial diversity and richness, such as during habitat restoration after

459 contaminated soil (Yin et al., 2000; Harris, 2003). Fungal diversity was higher for the pre-
460 droughted than the pre-sterilised soil (but did not differ from the control).

461

462 **4.2. Impact of drought on microbial-community composition**

463 The drought treatment generally had no impact on total microbial biomass, which may seem
464 surprising, but previous studies have also reported mixed results. For example, an
465 experimental drought treatment actually increased microbial biomass in a mountain meadow
466 (Fuchslueger et al., 2014), hypothesised to be due to the continuation of carbon inputs from
467 plants during the drought, especially to fungi. Similarly, a study of two grasses and one
468 leguminous species recorded higher microbial biomass under drought when plants were
469 grown in mixtures and variable trends under monocultures (Sanaullah et al., 2011), and a 10-
470 month throughfall-exclusion experiment in a tropical forest found no effect on microbial
471 biomass (Bouskill et al., 2013). Others studies, however, have reported a decrease in
472 microbial biomass linked to lower soil water content, such as in a six-year drought
473 experiment in a semiarid forest (Bastida et al., 2017), in a short-term experiment without
474 plants (Chowdhury et al., 2011), and in a hardwood forest correlating natural variation in soil
475 moisture with soil microbial biomass (Baldrian et al., 2010). Importantly, total microbial
476 biomass in our study was lower in the pre-droughted than the control soils when the plant was
477 present, providing evidence that the long-term drought conditions damaged the soil microbial
478 community, rather than led to acclimation as is sometimes hypothesised. Soils with an
479 associated plant community represent a more realistic scenario than a bare, non-vegetated
480 soil, and this result may indicate that only a small proportion of the soil microbiota are able to
481 adapt to the drought conditions (Kaisermann et al., 2017).

Drought duration had a significant effect on the fungal:bacterial ratio, which increased with increasing drought. This result is broadly consistent with the majority of previous studies on this topic (Bapiri et al., 2010; Barnard et al., 2015). It is likely attributable to the chitinous cell walls of fungi, which should increase their resistance to environmental fluctuations, such as water stress (Holland and Coleman, 1987), and fungal hyphal growth (which most bacteria do not have) allowing them to cross small areas of dry soil (Yuste et al., 2011). Trends in fungal versus bacterial dominance, however, are variable and may depend on the trait measured (e.g. biomass or growth) and the method used (Strickland and Rousk, 2010). Drought also decreased the ratio of Gram-positive:Gram-negative bacteria, which was surprising because Gram-positive bacteria are typically more drought resistant (Schimel et al., 2007). This trait, however, may be linked to the increase in root exudation by *Q. ilex* during increasing drought (Preece et al., 2018), because Gram-negative bacteria preferentially consume this type of labile carbon source, whereas Gram-positive bacteria tend to consume more recalcitrant C sources (Balasooriya et al., 2014; Naylor and Coleman-Derr, 2017).

In addition to reducing the proportion of bacterial biomass compared to fungi, drought also clearly affected the composition of the bacterial community. Drought increased the relative abundance of *Actinobacteria*, supporting previous studies that also reported this same pattern in a range of soils and in the rhizosphere and endosphere of various plant species (Bouskill et al., 2013; Nessner Kavamura et al., 2013; Naylor and Coleman-Derr, 2017). This increase in abundance may be due to the ability of *Actinobacteria* to form spores, which would allow them enter a dormant state during periods of environmental stress, such as drought (Naylor and Coleman-Derr, 2017; Taketani et al., 2017).

The comparison of community compositions did not reveal an overall impact of drought on fungal-community composition, but drought affected specific functional groups, although these effects depended on soil history. For example, the relative abundance of plant

pathogens increased during drought in the control soils but decreased in the pre-droughted and pre-sterilised soils. *Phytophthora* diseases such as *P. cinnamomi* are generally favoured on soils where drainage is impeded (Desprez-Loustau et al., 2006) and have been shown to increase in soils during adverse climatic conditions (such as drought or waterlogging) due to host plants becoming less stress resistant, allowing a build up of pathogens in the soil (Brasier, 1996; de Sampaio e Paiva Camilo-Alves et al., 2013). However, contrary to our findings, most soil pathogens are thought to be favoured by wetter soils (Cook and Papendick, 1972).

In addition, the relative abundance of yeasts was positively correlated with drought duration in the pre-sterilised soil but decreased in the pre-droughted soil and did not change in the control soil. This result suggests a complex network of interactions between environmental factors (in this case water stress) and the pre-existing soil community, where a difference in the history of the soil can generate variation in the subsequent response of fungal community composition to perturbation. Previous research has predicted a potential increase in yeasts under future drought scenarios, as they tend to be found in more stressful environments (Treseder and Lennon, 2015).

4.3. Impact of drought on microbial-community diversity

The experimental drought generally had a negative effect on bacterial-community alpha diversity (Shannon H and Simpson index), which has also been reported in a previous drought study (Bouskill et al., 2013), although diversity is generally not affected (Bachar et al., 2010; Acosta-Martínez et al., 2014; Naylor and Coleman-Derr, 2017; Tóth et al., 2017). Drought had positive effects on fungal diversity, specifically in the low- and high-level drought treatments compared to the control. Previous studies have reported higher fungal

diversity under drought (Acosta-Martínez et al., 2014; Schmidt et al., 2018) and may indicate a higher tolerance of these organisms to drought, which would allow them to thrive if bacteria are negatively affected.

In addition to the impact of drought on diversity, we noticed a clear pattern in the effect of plants and soil. Not surprisingly, both bacterial and fungal diversity were lower in the pre-sterilised soil than the other two soil histories, as communities in pre-sterilised soil were much more recently created, with only a short time for colonisation of microorganisms. Bacterial diversity was higher in the pre-droughted soil, indicating a positive effect of the past precipitation regime. This agrees with a previous study that also found a positive legacy effect of previous drought on bacterial diversity in a tropical forest soil (Bouskill et al., 2013). The presence of the plant was very beneficial in augmenting both bacterial and fungal diversity in the soil with the lowest diversity (pre-sterilised soil). This was almost certainly due to the presence of microbes on the roots of the saplings at planting, which would have had much more of an impact in this soil history type. Plant presence has been shown to have beneficial effects on bacterial diversity, such as in a previous study in a semiarid shrub system which found higher Shannon index, evenness and richness under the two shrub species than under bare soil (Hortal et al., 2015).

When interpreting the effects of drought duration on the measured responses it is important to note that samples for different drought duration treatments were taken at different time points. It is therefore possible that underlying temporal dynamics, perhaps related to disturbance during soil sampling, and therefore unrelated to the treatment, could be causing the observed patterns. However, given the extended pre-incubation and stabilization period of six weeks prior to the initiation of drought, and the fact that disturbance effects on microbial community in incubations attenuate over relatively short time periods (e.g. Weedon et al., 2013) we consider it unlikely that such temporal dynamics would be as large as drought

effects. It is also important to note that the interpretation of alpha diversity measures from amplicon data can be problematic due to potentially spurious OTUs and the possibility of some taxa falling under the detection limit due to incomplete sampling. These results should therefore be considered as preliminary and be used as the basis for more detailed future studies.

4.4. Are bacteria more affected than fungi by drought?

Drought tended to affect the community composition of bacteria more than fungi, and the proportion of bacterial biomass compared with fungal biomass decreased under water stress, even though total microbial biomass was unaffected by drought. Taken together, this could suggest important changes in the future functioning of the bacterial community in soils exposed to water stress, for example relating to carbon and nutrient cycling (Schimel et al., 2007; Frank et al., 2015). During drought (in water-limited areas) decomposition rates slow, leading to a build up in soil organic matter (SOM) and lower N mineralisation (Borken and Matzner, 2009; Larsen et al., 2011; van der Molen et al., 2011; Sanaullah et al., 2012; Nguyen et al., 2018). The lack of strong effects of drought on fungal-community composition may be due to different responses being found depending on the strength of the drought, thus a lack of a clear unidirectional pattern: fungal diversity increased under low- and high-level drought but not mid-level drought. Soil bacteria are more abundant than fungi, so fungi may tend to take advantage of gaps where and when they can, resulting in a less standardised and more idiosyncratic response to disturbance. For example, we could speculate that under low drought there is some release of competition with bacteria, but during mid-level drought this is offset by inhibitive effects, such as low substrate diffusion rates and energetically expensive solute accumulation (Schimel et al., 2007). During extreme drought, some fungi

will benefit from being able to consume necromass, and this may especially favour fungi if there is an increase in the C:N ratio of this dead mass (Moore et al., 2004) which can happen under drought (Crowther et al., 2015). In addition, the bacterial community is able to respond much quicker than fungi to the experimental conditions, thus it could be that bacteria are also more rapidly affected by the imposed drought, and such responses are slower to be seen in fungi. This would fit with previous studies that have found that soil bacterial activity was more responsive to soil water content than fungal activity (Bell et al., 2008) and that changes to bacterial communities under drought were longer lasting than for fungal communities (de Vries et al., 2018). However, it is difficult to generalise across other systems, and there is some evidence indicating that fungi are more sensitive than bacteria to smaller changes in soil moisture (Kaisermann et al., 2015) with some cases where fungal abundance was more greatly reduced by drought (Cregger et al., 2012).

4.5. Does the presence of *Q. ilex* lessen the impacts of drought on microbial communities?

Previous studies have reported strong links between above and belowground communities, and high plant diversity tends to increase soil microbial biomass and activity due to inputs of organic matter and the regulation of soil moisture (Zak et al., 2003; Lange et al., 2015; Thakur et al., 2015). The presence of plants can also shield microbial communities from the impacts of drought (de Vries et al., 2012) and deep roots may act as moisture hotspots during dry seasons (Castaño et al., 2018). We hypothesised that the presence of *Q. ilex* would be beneficial for microbes (e.g. total microbial biomass), because plants are a source of carbon inputs, such as root exudates and litter that provide a readily available energy source for many microorganisms (Dennis et al., 2010). Indeed, a recent study of root exudation by our

study species, *Q. ilex*, under the same experimental conditions found that the exudation of carbon increased during drought (Preece et al., 2018). Additionally, a review summarising published drought impacts on root exudation in a range of species and with various experimental methods found that carbon inputs tend to increase under drought, although this effect may decrease or reverse under very severe drought (Preece and Peñuelas, 2016). The impact of water stress on the overall composition and diversity of both bacteria and fungi in our current study, however, was not affected by the presence of *Q. ilex*. Whilst we must be careful about the conclusions drawn from amplicon data about alpha diversity, these results suggest that any protective effect of the presence of the plant on microbial diversity would be very minor, especially in comparison to other factors such as water stress or soil history.

Water is so limiting to microbial growth (Manzoni et al., 2011) that any positive effect of the plant under drought conditions of more than a few days was likely minimal. Although without measurements of water potential this is hard to confirm. An increase in carbon inputs to the soil by plants also likely only affects a very isolated area near the roots (Dennis et al., 2010), and these inputs cannot easily diffuse in dry soil to be able to influence a large proportion of the soil microbes. An additional consideration is that the length of this experiment may not have been sufficient for demonstrating the full impacts on fungi, which have relatively slow growth compared to bacteria. For example, bacterial turnover time tends to be days to weeks, whereas fungal turnover is usually weeks to months (Rousk and Bååth, 2011). The complexity of the microbial community may also mask some impacts that affect some groups of the community. Many specific bacterial and fungal taxa showed an interaction between the drought treatment and the presence of *Q. ilex*, although the direction of the responses was not consistent. For example, *Gammaproteobacteria* relative abundance was negatively correlated with increasing drought when *Q. ilex* was absent, but there was no

correlation when *Q. ilex* was present, whereas *Cytophagia* showed the opposite pattern (negative correlation with drought only when *Q. ilex* was present).

It is important to note that although the roots of the saplings were thoroughly cleaned before planting, it was impossible to completely remove the original community present on the roots. This community was from the substrate that the plants were grown in at the nursery they were purchased from, and could have influenced the microbial community that we measured. However, this should not affect our interpretation of any interaction between the presence of *Q. ilex* and the drought treatment as the impact of roots being present (with all of the accompanying rhizosphere microbes and root processes) versus roots being absent, would be much greater than any small variations in rhizosphere composition due to the amount of influence of the initial community. Additionally, all roots were treated equally, so there should be no systematic difference in the influence of the initial inoculum for the different plants.

4.6. Are soil microbial communities with a history of drought more resistant to drought?

Recent studies have identified 'legacy effects' of drought-stressed soils. This means that for soils that have been previously water stressed, a subsequent drought may have a stronger or weaker impact on the soil community than a soil without such a history (de Vries et al., 2012; Evans and Wallenstein, 2012; Bouskill et al., 2013; Hawkes and Keitt, 2015; Kaisermann et al., 2017; Meisner et al., 2018). A stronger impact could be due to the loss of resistance or resilience of a repeatedly disturbed soil, whereas a weaker impact would imply the selection of taxa that are better adapted to the conditions. We found no interaction between drought and soil history for the overall bacterial or fungal composition or diversity, but some

652 evidence suggested that previous soil history affected the response of specific taxa to drought.
653 For example, the relative abundance of the bacterial phylum *Chloroflexi* was correlated
654 positively with drought duration in the control soil but negatively in the pre-droughted soil.
655 Relative abundance may thus increase during a short-term drought (the current experiment)
656 but not during long-term perturbations (the 16 years of the long-term drought study).
657 Previous studies have found *Chloroflexi* to increase in relative abundance during drought
658 periods or increasing aridity (Acosta-Martínez et al., 2014; Maestre et al., 2015). However,
659 this is not a universal response, with another recent study finding a decrease in relative
660 abundance under drought (Meisner et al., 2018).

661 Another interesting example is the bacterial class *Thermoleophilia*, where diversity
662 (number of OTUs) was positively correlated with drought duration, but only in the pre-
663 droughted soil. The abundance of *Thermoleophilia* was also positively correlated with
664 drought duration (in the control and pre-droughted soils) and was therefore an example of a
665 group of bacteria that may be able to take advantage of recurrent droughts, consistent with
666 previous studies reporting that *Thermoleophilia* can respond positively to drought (Pereira de
667 Castro et al., 2016; Ochoa-Hueso et al., 2018). *Thermoleophilia* diversity, however, was not
668 higher in the pre-droughted soil before the start of the current drought experiment,
669 demonstrating the difficulty in both predicting the response of soil microbes and
670 understanding the mechanisms behind any adaptation to drought in long-term droughted soil.

671 Bacterial and fungal alpha diversity before the start of the drought treatment was
672 highest in the pre-droughted soil, which could indicate a source of resilience or resistance for
673 the microbial community, even though the diversity was not significantly higher than in the
674 control soil. Further investigation of this finding may provide evidence of adaptation to water
675 stress. Our results indicate a tendency for plant fungal pathogens to increase under drought,
676 and have a higher abundance in soils with a long-term history of drought. This may be due to

677 the damaging impact of drought on plants, which may increase their susceptibility to disease
678 and thereby increase the population of fungal soil pathogens (Brasier, 1996). Pre-droughting
679 had a negative legacy effect on the saprotrophic yeasts, with relative abundance negatively
680 correlated with drought duration in the pre-droughted soil. The abundance of yeasts in this
681 case was not higher in the pre-droughted soil, which may indicate that the long-term
682 historical drought increased the vulnerability of this group to the subsequent drought in this
683 experiment.

684 The soil inoculum used in this study was taken from a Mediterranean holm oak forest,
685 which is exposed to relatively large variation in precipitation within and between years
686 (Ogaya and Peñuelas, 2007; Liu et al., 2015). In the period from 1999-2015, mean annual
687 precipitation was 616.1 mm, and this varied greatly from 379.8 mm (in 2006) to 926.7 mm
688 (in 2010). The majority of rainfall (80%) is in spring (March-May) and autumn (September-
689 November), with less than 10% in summer (June-August). The soil water content varies
690 between ~10% v/v in summer to ~30% v/v in spring and autumn. Whilst the long-term
691 drought treatment did decrease mean soil moisture throughout the study period by 13%
692 compared with control plots (Liu et al., 2015), the high variability in precipitation may reduce
693 the chance of the microbial community demonstrating legacy effects, as the soil can cycle
694 between being very dry and then saturated, and the 'control' soil community may already be
695 dominated by phenotypes that can tolerate the dry summer conditions (Curiel Yuste et al.,
696 2014). Thus legacy effects may be less pronounced in this Mediterranean system compared
697 with areas that have more uniform precipitation patterns, such as in temperate locations (de
698 Vries et al., 2012; Kaisermann et al., 2017; Meisner et al., 2018), humid continental (Evans
699 and Wallenstein, 2012), or humid tropical sites (Bouskill et al., 2013).

700

4.7. The future of soil communities under drought

Drought is a growing threat around the world, and we have demonstrated complex effects on bacterial and fungal communities that depend on the intensity of the drought, the presence of plants, and previous soil history. We have particularly demonstrated that bacteria may be more negatively affected than fungi in terms of biomass stock and that plants may provide some protection for maintaining microbial diversity, so bare soils may be more at risk. Soils with a history of long-term drought showed a legacy effect, which positively affected the diversity of the bacterial community, presumably due to the adaptation of the soil community to these conditions. We also found, however, many examples of taxa or functional groups with a negative legacy effect due to the historical drought. Also it might indeed be that the negative effect on some taxa represents an alleviation of competition that leaves resources available to other taxa, which in turn can increase the diversity. We therefore cannot assume that soil communities will be able to adapt to the occurrence of more frequent or severe droughts and continue to maintain the same functions. Drought will continue to have impacts on microbial community composition, with a general shift towards an increasing proportion of fungi and a decrease in the mass and diversity of bacteria.

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	Bacteria						Fungi					
Treatment factor	**Composition**		**Shannon diversity**		**Simpson index**		**Composition**		**Shannon diversity**		**Simpson index**	
	R^2	*P*	χ^2	*P*	χ^2	*P*	R^2	*P*	χ^2	*P*	χ^2	*P*
Soil history	0.25	***	NS	NS	6.3	*	0.51	***	11.9	**	NS	NS
Plant	0.16	***	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Soil history * Plant	NS	NS	29.5	***	21.9	***	NS	NS	NS	NS	9.3	**

Table 1. Factors affecting initial differences in soil community composition (results of PERMANOVA using the *adonis2* function in the R *vegan* package) and Shannon diversity (results of linear mixed effects model). Asterisks represent the *P*-value:* = *P* < 0.05, ** = *P* < 0.01, * = *P* < 0.001. NS = non-significant.**

Treatment factor	**Bacteria**				**Fungi**			
Type of drought variable	Continuous		Factor		Continuous		Factor	
	R^2	*P*	R^2	*P*	R^2	*P*	R^2	*P*
Drought	0.12	***	0.06	***	NS	NS	NS	NS
Soil history	0.17	***	0.17	***	0.40	***	0.40	***
Plant	0.08	***	0.08	***	0.04	***	0.04	***
Drought * Soil	NS	NS	NS	NS	NS	NS	NS	NS
Drought * Plant	NS	NS	0.03	*	NS	NS	NS	NS
Soil history * Plant	0.03	***	0.03	***	0.03	*	0.03	*

Table 2. Results of the PERMANOVA of microbial composition (Bray-Curtis dissimilarity indices) using the adonis2 function in the R vegan package. Drought was treated as both a continuous variable (days of drought) and as a factor (control, low, mid, high), as the impact of drought may not always be linear. Asterisks represent the *P*-value: * = *P* < 0.05 and * = *P* < 0.001.**

	Bacteria				**Fungi**			
	Shannon		**Simpson**		**Shannon**		**Simpson**	
Factor	χ^2	***P***	χ^2	***P***	χ^2	***P***	χ^2	***P***
Drought	9.0	**	6.2	*	21.6	***	19.0	***
Soil history	12.7	**	NS	NS	15.4	***	NS	NS
Plant	NS	NS	NS	NS	NS	NS	NS	NS
Soil history * plant	37.6	***	19.4	***	9.0	*	13.6	**

Table 3. Effect of drought, soil history, and the presence of *Q. ilex* on the Shannon and Simpson index of bacteria and fungi. Drought duration is a continuous variable for bacteria and a categorical variable for fungi as the response to drought was non-linear.

Soil history	*Q. ilex*	ΔH	
		Bacteria	Fungi
Control	Present	-2.91	+26.04
Control	Absent	-1.71	+8.33
Pre-droughted	Present	-1.58	-1.46
Pre-droughted	Absent	-1.84	+17.47
Pre-sterilised	Present	-4.08	+15.15
Pre-sterilised	Absent	-3.94	+34.65

Table 4. ΔH (% change after most intense drought compared to control) of the Shannon diversity (H) of the bacterial and fungal communities to drought.

Fig. 1

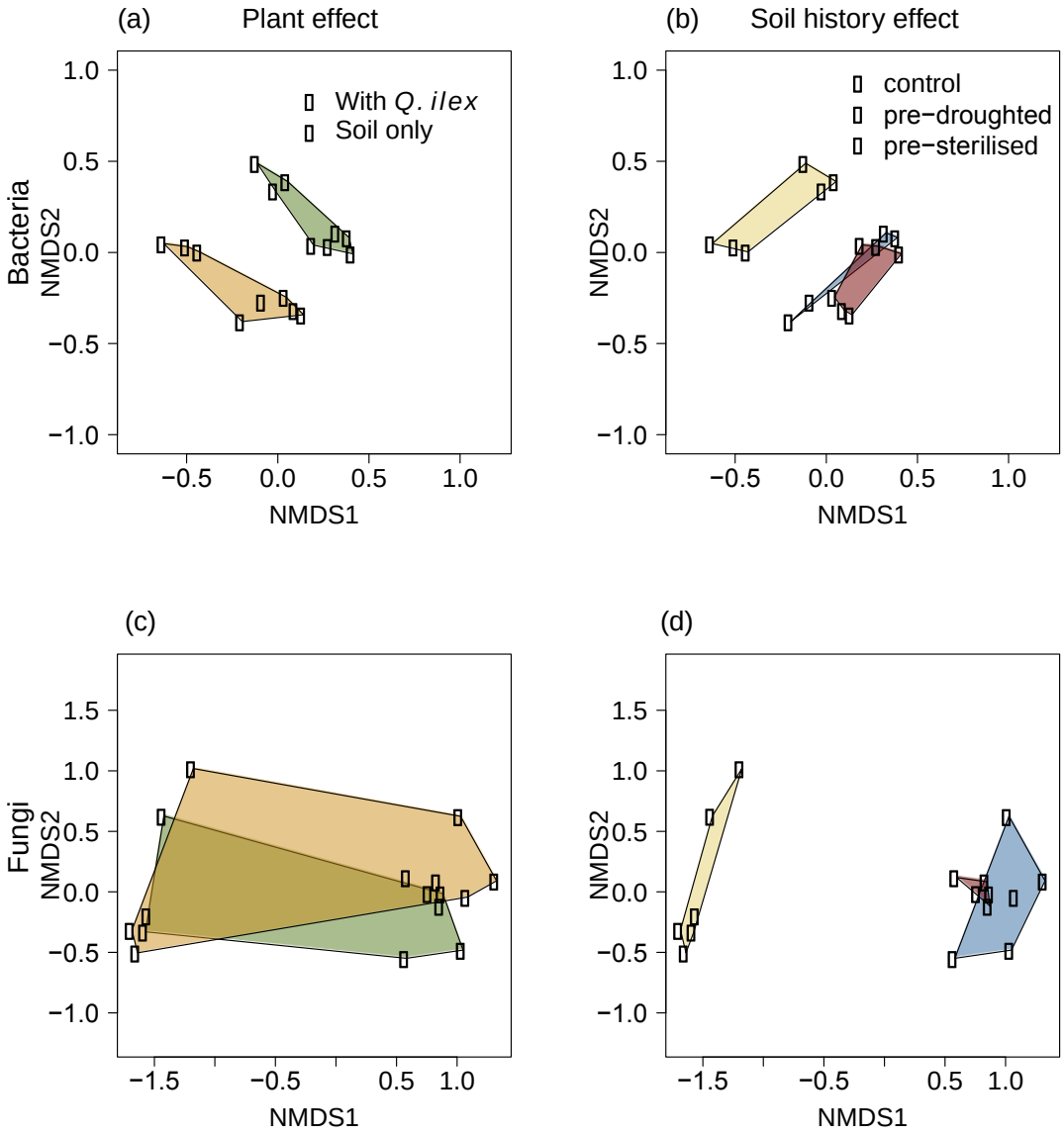

Fig. 1. NMDS plots based on Bray-Curtis dissimilarities of the (a,b) bacterial and (c,d) fungal communities prior to onset of drought (drought duration of zero days), showing the difference due to the presence of *Q. ilex* and the soil history. Points represent individual samples, convex hulls encompass samples of the same treatment.

Fig. 2

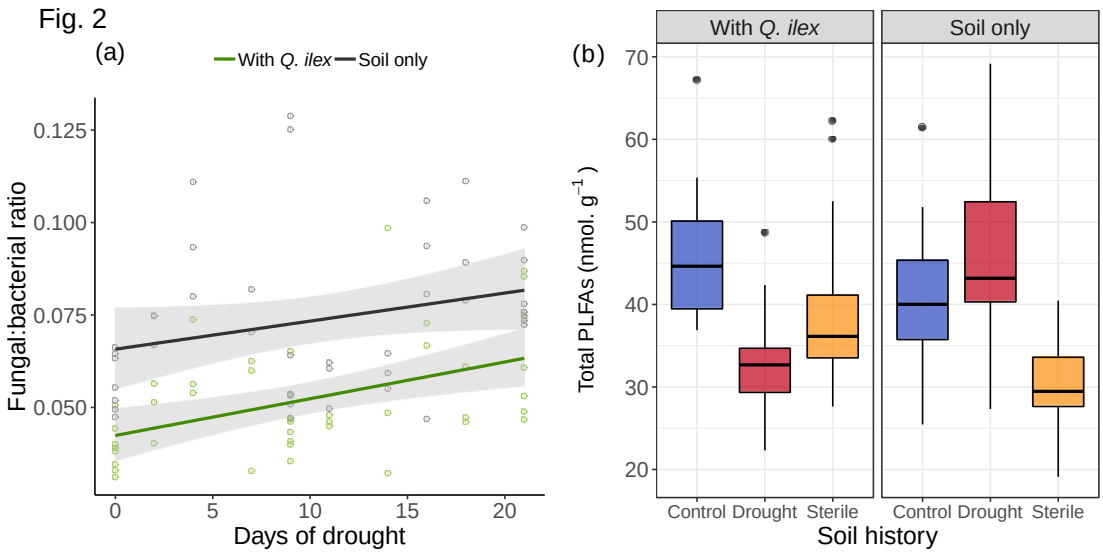

Fig. 2. (a) Fungal:bacterial ratio for the drought durations with *Q. ilex* present (green lines and dots) and for soil only (black lines and grey dots) There was a positive relationship with drought ($P < 0.01$) and a positive effect of plant-presence ($P < 0.001$). (b) Total PLFAs (nmol g^{-1}) separated by soil history and presence of *Q. ilex* (blue) or soil only (red). n = 15 for each plant and soil combination. Soil treatments are 'Control', 'Drought' (pre-droughted) and 'Sterile' (pre-sterilised).

Fig. 3

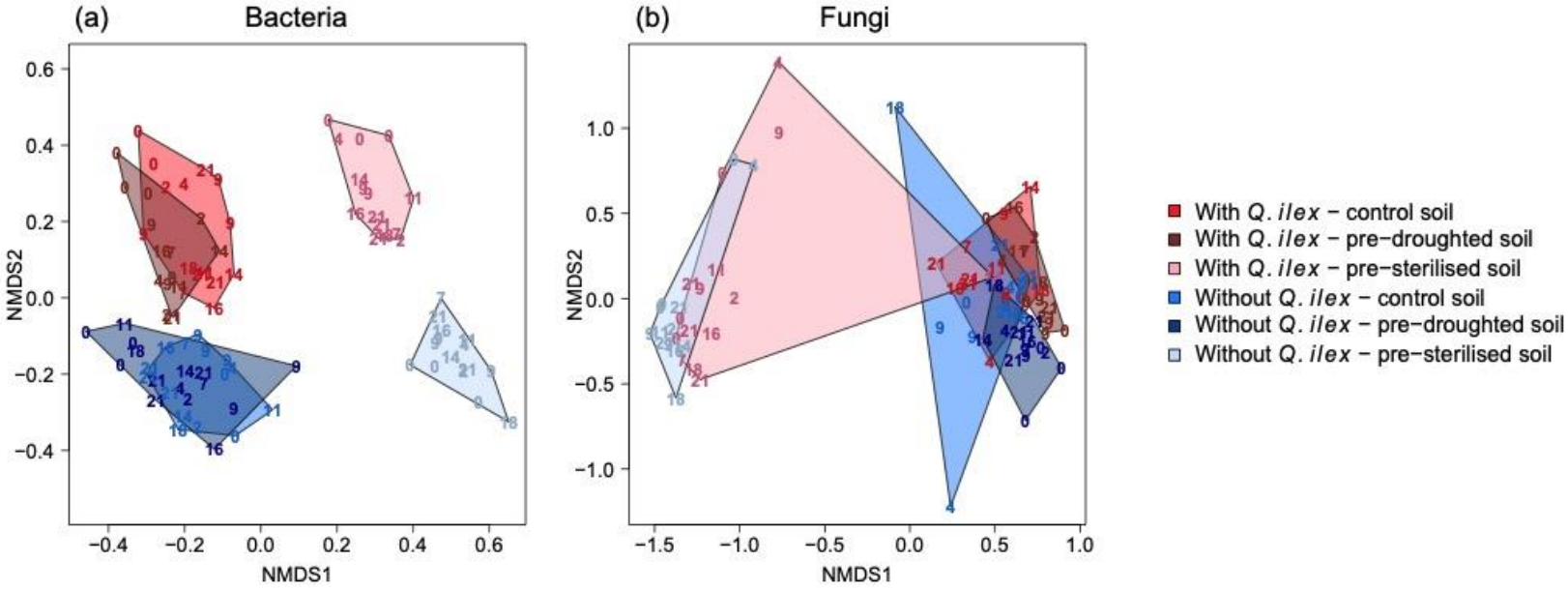

Fig. 3. NMDS plots based on Bray-Curtis dissimilarities of the (a) bacterial and (b) fungal communities after drought treatments of 0 to 21 days. Numbers (0 to 21) show the length in days of the drought treatment, and represent individual samples. The six combinations of soil history and plant presence are grouped by colour, with shades of red when *Q. ilex* was present and shades of blue when *Q. ilex* was absent. Convex hulls encompass samples of the same treatment.

Fig. 4

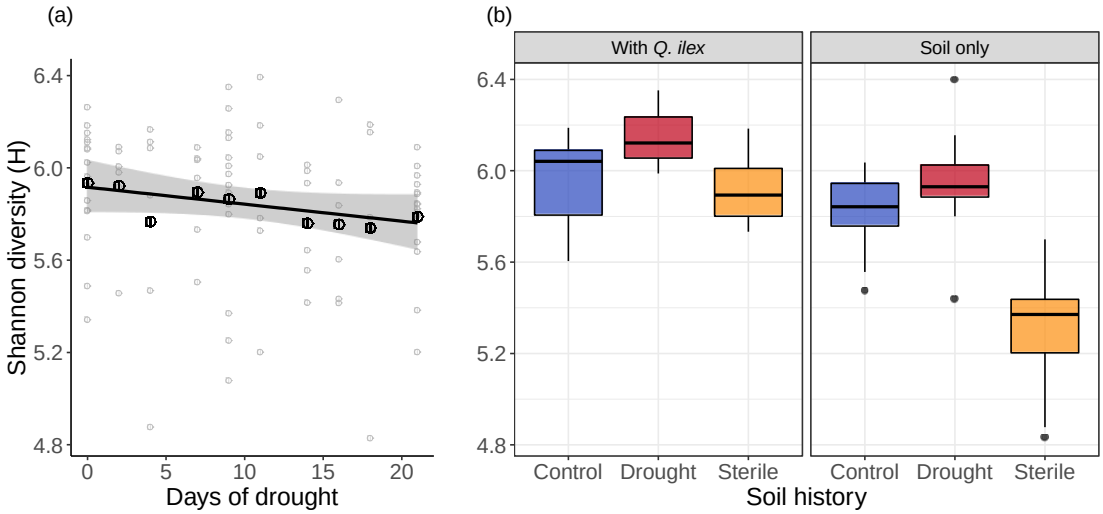

Fig. 4. Relationships between bacterial Shannon diversity and (a) drought duration (days) ($P < 0.001$) and (b) soil history ($P < 0.01$, all three soil histories differed from each other) and the presence of *Q. ilex* (significant interaction, $P < 0.001$). n = 15 for each plant and soil combination. Soil treatments are 'Control', 'Drought' (pre-droughted) and 'Sterile' (pre-sterilised). For plot (a) small grey points represent individual samples and larger black points are mean values.

Fig. 5

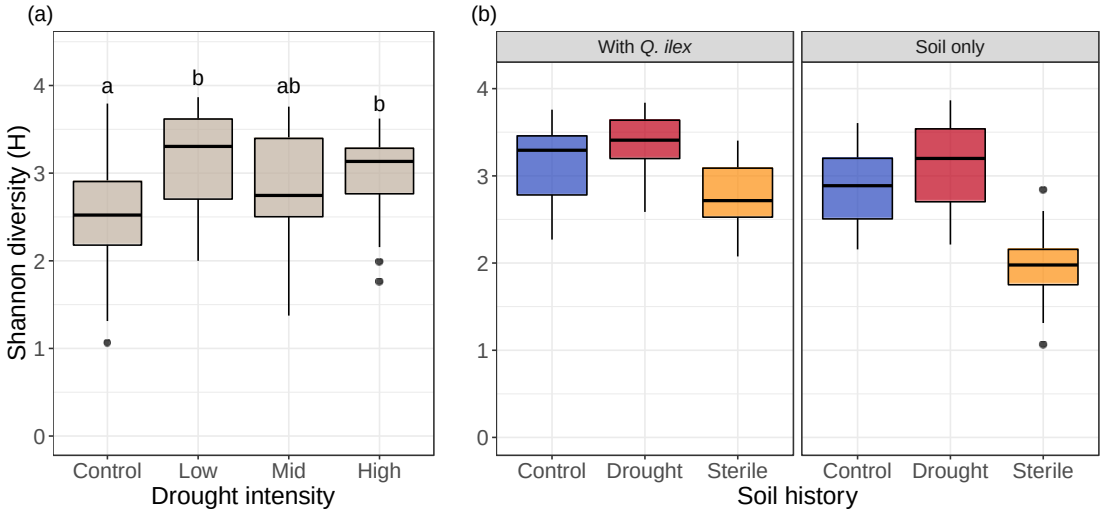

Fig. 5. Relationships between fungal Shannon diversity and (a) drought duration as a categorical variable (Control, 0 days of drought; Low, 2-7 days; Mid, 9-14 days; High, 16-21 days; $P < 0.001$) and (b) soil history ($P < 0.01$, pre-sterilised soil differs from the control and pre-droughted soil) and the presence of *Q. ilex* (significant interaction, $P < 0.001$). Letters a and b denote treatments which differ from each other (Tukey HSD test, $P < 0.05$). n = 15 for each plant and soil combination except those with *Q. ilex* and in pre-droughted soil, where n = 14. Soil treatments are 'Control', 'Drought' (pre-droughted) and 'Sterile' (pre-sterilised).

Supporting Information for:

Effects of past and current drought on the composition and diversity of soil microbial communities

Catherine Preece, Erik Verbruggen, Lei Liu, James Weedon, Josep Peñuelas.

Drought duration (d)	Soil only			With Plant		
	Control	Drought	Sterile	Control	Drought	Sterile
	n	**n**	**n**	**n**	**n**	**n**
0	2	3	3	2	3	3
2	1	1	1	1	1	1
4	1	1	1	1	1	1
7	1	1	1	1	1	1
9	3	2	3	3	3	2
11	1	1	1	1	1	1
14	1	1	1	1	1*	1
16	1	1	1	1	1	1
18	1	1	1	1	1**	1
21	3	3	2	3	2	3

Table S1. The number of replicates shown for each combination of drought, soil and plant treatment. * For the fungi, this sample was not able to be used, thus there was no replicate for the fungal analyses. ** This sample was not able to be used for either bacteria or fungi.

Treatment factor	**Bacteria**				**Fungi**			
Type of drought variable	Continuous		Factor		Continuous		Factor	
	R^2	*P*	R^2	*P*	R^2	*P*	R^2	*P*
Drought	0.19	***	0.09	***	NS	NS	0.07	*
Soil history	0.05	***	0.05	***	0.09	***	0.10	***
Plant	0.09	***	0.09	***	0.10	***	0.10	***
Drought * Soil history	NS	NS	NS	NS	NS	NS	NS	NS
Drought * Plant	NS	NS	0.05	*	NS	NS	NS	NS
Soil history * Plant	0.02	**	0.02	*	NS	NS	NS	NS

Table S2. Results of the PERMANOVA of microbial composition (Bray-Curtis dissimilarity indices) when the pre-sterilised soil was not included. The adonis2 function in the R vegan Package was used. Drought was treated as both a continuous variable (days of drought) and as a factor (control, low, mid, high), as the impact of drought may not always be linear. Asterisks represent the *P*-value: * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$.

	Pairs	n	*F*	R^2	*P* adj.
Bacteria	$D_{con} - D_{low}$		1.65	0.049	0.198
	$D_{con} - D_{mid}$		2.14	0.049	0.018
	$D_{con} - D_{high}$		2.55	0.059	**0.006**
	$D_{low} - D_{mid}$		1.00	0.022	1.000
	$D_{low} - D_{high}$		1.49	0.033	0.366
	$D_{mid} - D_{high}$		2.04	0.037	0.060
Fungi	$D_{con} - D_{low}$		0.86	0.028	1.000
	$D_{con} - D_{mid}$		0.69	0.017	1.000
	$D_{con} - D_{high}$		0.93	0.022	1.000
	$D_{low} - D_{mid}$		0.96	0.023	1.000
	$D_{low} - D_{high}$		0.82	0.019	1.000
	$D_{mid} - D_{high}$		0.93	0.018	1.000

Table S3. Results of the pairwise PERMANOVA plots of microbial composition (Bray-Curtis dissimilarity indices) between different drought levels (with drought as a factor). Drought levels are: D_{con} = control (0 days drought), D_{low} = low (2-7 days drought), D_{mid} = mid (9-14 days drought), D_{high} = high (16-21 days drought).

	Pairs	n	*F*	R^2	*P* adj.
Bacteria	$S_{con} - S_{dro}$		2.93	0.049	**0.003**
	$S_{con} - S_{ste}$		11.76	0.169	**0.003**
	$S_{dro} - S_{ste}$		12.16	0.176	**0.003**
Fungi	$S_{con} - S_{dro}$		5.95	0.096	**0.003**
	$S_{con} - S_{ste}$		33.65	0.384	**0.003**
	$S_{dro} - S_{ste}$		40.76	0.421	**0.003**

Table S4. Results of pairwise PERMANOVA of microbial composition (Bray-Curtis dissimilarity indices) between different soil histories. Soil histories are: S_{con} = control, S_{dro} = pre-droughted, S_{ste} = pre-sterilised.

Phylum	**Change in relative abundance**					
	Drought * Soil history	**Drought * Plant**	**Plant * Soil history**	**Drought effect**	**Soil history effect**	**Plant effect**
Proteobacteria	NS	NS	NS	-ve correlation	pre-sterilised soil > control = drought	soil only < with *Q. ilex*
Actinobacteria	NS	NS	NS	+ve correlation	pre-sterilised soil < control = drought	soil only > with *Q. ilex*
Bacteroidetes	-ve correlation in control soil and no correlation in pre-sterilised soil	NS	soil only in pre-sterilised soil > other combinations	NS	NS	NS
Verrucomicrobia	NS	NS		NS	pre-sterilised > control	soil only < with *Q. ilex*.
Planctomycetes	NS	NS	in soil only, pre-sterilised soil < control = drought, and soil only with pre-sterilised soil < with *Q. ilex* in drought soil	+ve correlation	NS	NS
Chloroflexi	+ve correlation in control soil, -ve correlation in pre-droughted soil	NS	in soil only, pre-sterilised < control = drought; with *Q. ilex*, pre-sterilised = control < drought	NS	NS	NS
Acidobacteria	NS	NS	soil only in pre-sterilised soil > other combinations	NS	NS	NS

Firmicutes	NS	+ve correlation when *Q. ilex* present, -ve correlation in soil only	NS	NS	pre-sterilised > control = drought	NS

Table S5. Summary of differences in relative abundances of bacterial phyla contributing > 2% of total reads during the drought experiment. NS, not significant. +ve = positive and =ve = negative.

Class	**Change in relative abundance**					
	Drought * Soil history	**Drought * Plant**	**Plant * Soil history**	**Drought effect**	**Soil history effect**	**Plant effect**
Alphaproteobacteria	NS	NS	NS	NS	NS	NS
Actinobacteria	NS	NS	NS	+ve correlation	NS	soil only > with *Q. ilex*
Betaproteobacteria	NS	NS	NS	NS	pre-sterilised > control = drought	NS
Gammaproteobacteria	NS	-ve correlation when soil only, no correlation when *Q. ilex* present	NS	NS	NS	NS
Saprospirae	NS	NS	soil only in pre-sterilised soil > other combinations	-ve correlation	NS	NS
Thermoleophilia	no correlation in pre-sterilised soil, +ve correlation in control and drought soils	NS	NS	NS	NS	NS
Cytophagia	+ve correlation in pre-sterilised soils, -ve correlation in control soils, no effect in drought soil	no correlation in soil only, -ve correlation when *Q. ilex* present	NS	NS	NS	NS
Deltaproteobacteria	NS	NS	+ve effect of *Q. ilex* in pre-sterilised soil	NS	NS	NS

Planctomycetia	NS	NS	NS	+ve correlation	NS	NS
Opitutae	NS	NS	+ve effect of *Q. ilex* in control soil	-ve correlation	NS	NS
Bacilli	NS	no correlation in soil only, +ve correlation when *Q. ilex* present	NS	NS	pre-sterilised soil > control = drought	NS
Anaerolineae	NS	NS	+ve effect of *Q. ilex* in pre-sterilised and drought	NS	NS	NS

Table S6. Summary of differences in relative abundances of bacterial classes contributing > 2% of total reads during the drought experiment. NS, not significant. +ve = positive and =ve = negative.

Functional group	Change in relative abundance					
	Drought * Soil history	**Drought * Plant**	**Plant * Soil history**	**Drought effect**	**Soil history effect**	**Plant effect**
Filamentous saprotrophs	NS	NS	NS	NS	pre-sterilised soil < control soil	soil only > with *Q. ilex*
Plant pathogens	-ve correlation in pre-sterilised and drought soil, +ve correlation in control soils	NS	NS	NS	NS	NS
Yeasts	+ve correlation in pre-sterilised soil, no correlation in control soil, -ve correlation in drought soil	NS	NS	NS	NS	soil only < with *Q. ilex*

Table S7. Summary of differences in relative abundance of fungal functional groups contributing > 1.5 % of total reads during the drought experiment. NS, not significant. +ve = positive and -ve = negative.

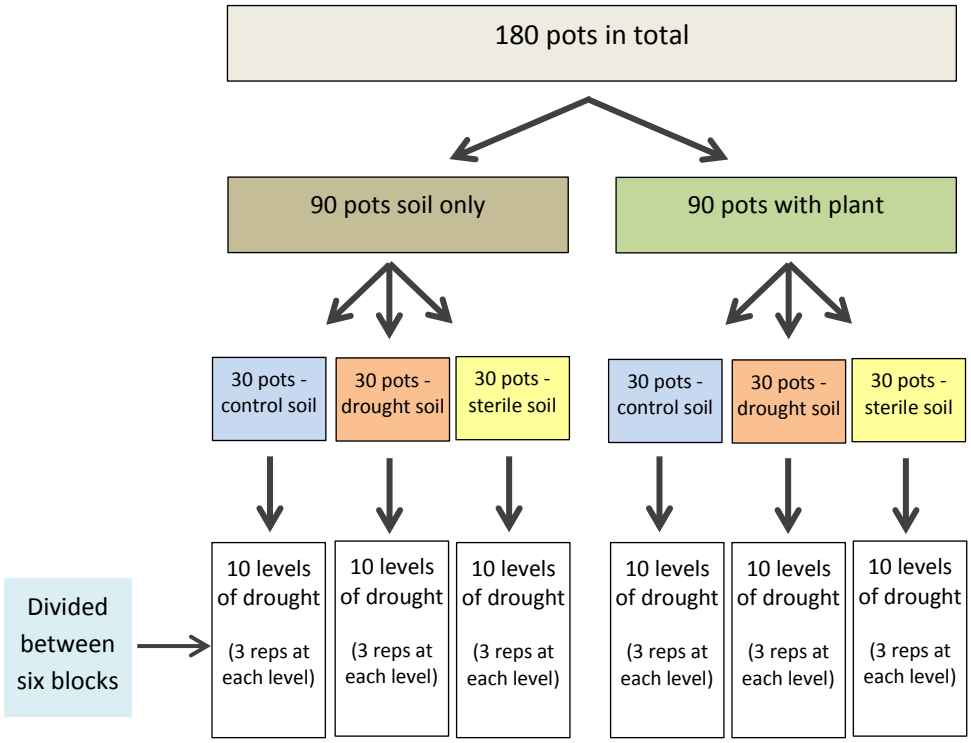

Fig. S1. Outline of the original experimental design, showing number of replicates in each treatment combination. Note that while there were three replicates of each treatment combination (plant × soil history × drought level) there were six experimental blocks. This was due to a second half of the experiment (not used in this article) which looked at the recovery following drought, thus doubling the number of replicates.

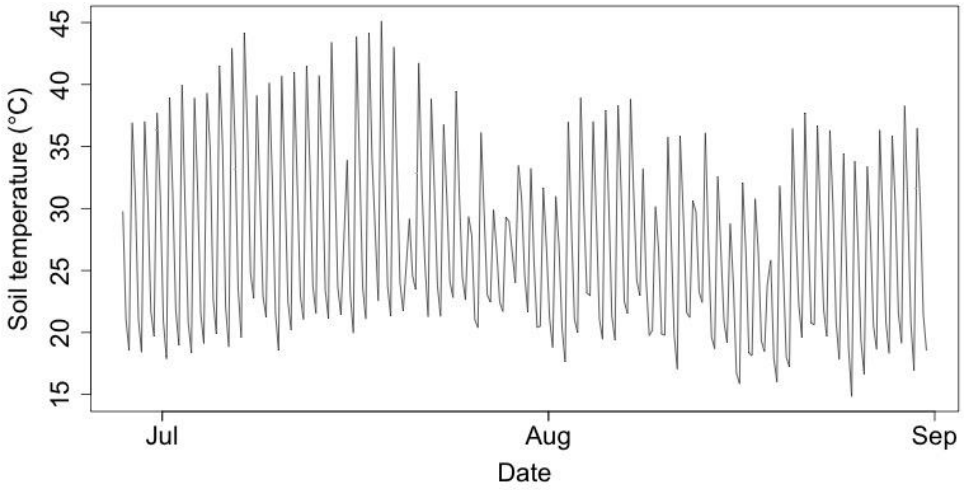

Fig. S2. Mean soil temperature throughout the experiment measured in five pots (due to availability of soil moisture probes).

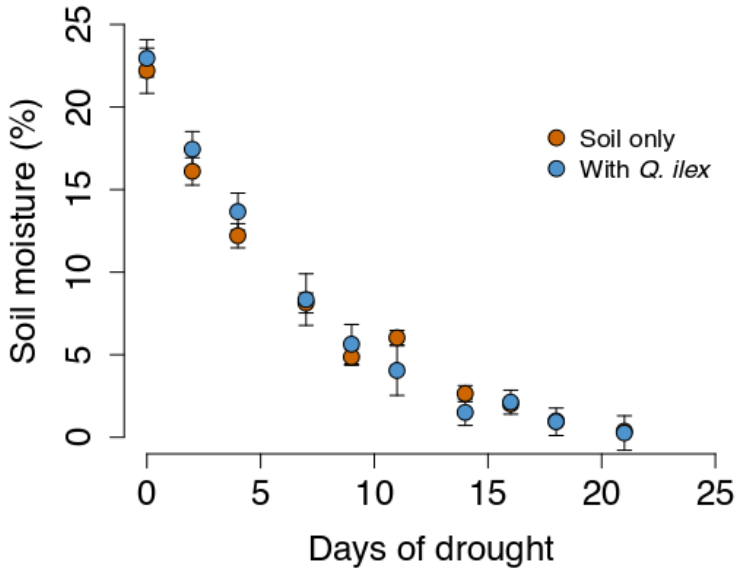

Fig. S3. Percentage soil moisture for the ten different drought levels measured at the end of each drought treatment. Points are means for each drought level, and shown in dark orange for pots without the plant ('soil only') and blue for pots with the plant ('with *Q. ilex*'). Standard error bars are shown. n = 18 at each drought measurement for each point, due to a second half of the experiment (not used in this article) which looked at the recovery following drought, thus doubling the number of replicates.

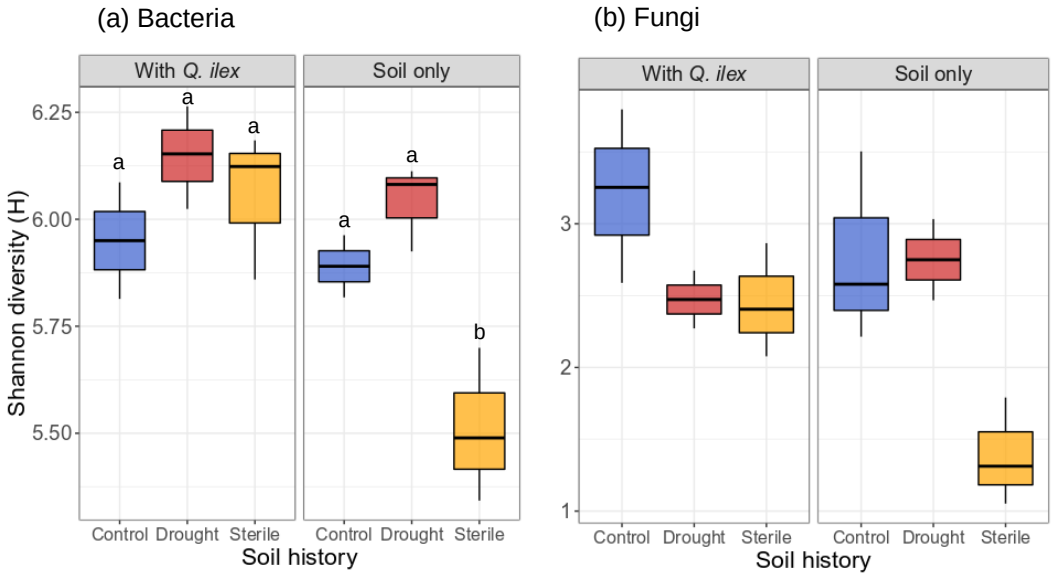

Fig. S4. Relationships between (a) bacterial and (b) fungal Shannon diversity and soil history and the presence of *Q. ilex*, prior to onset of drought (drought duration of zero days). Soil treatments are 'Control', 'Drought' (pre-droughted) and 'Sterile' (pre-sterilised). For bacteria there was a significant interaction ($P < 0.001$) between soil history and plant. Letters (a and b) signify significant ($P < 0.05$) differences following a Tukey test. For fungi there was a significant ($P < 0.05$) soil effect (pre-droughted higher than pre-sterilised).

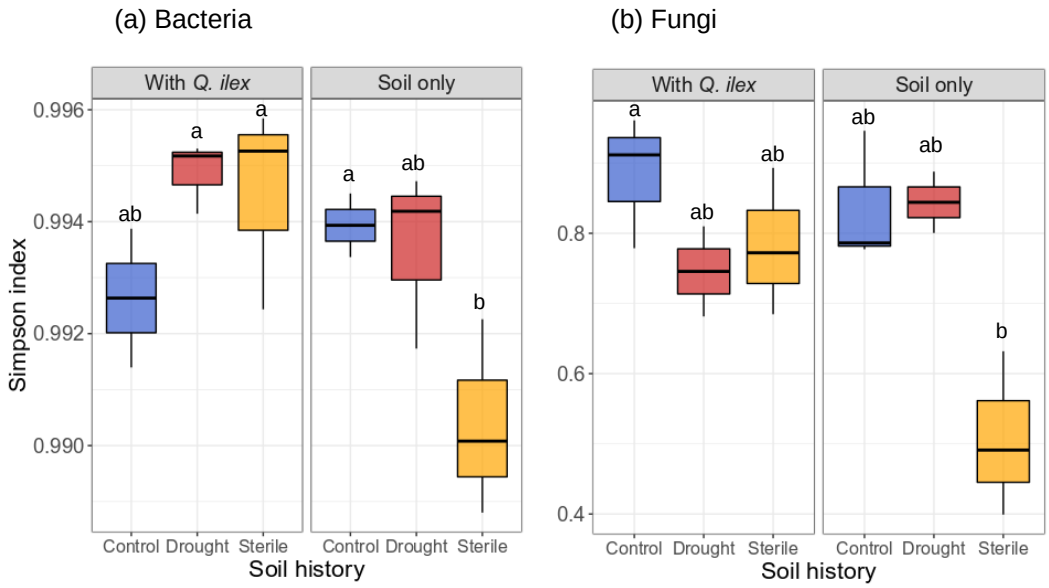

Fig. S5. Relationships between (a) bacterial and (b) fungal Simpson index and soil history and the presence of *Q. ilex*, prior to onset of drought (drought duration of zero days). Soil treatments are 'Control', 'Drought' (pre-droughted) and 'Sterile' (pre-sterilised). Letters (a and b) signify significant ($P < 0.05$) differences (within bacteria or fungi) following a Tukey test.

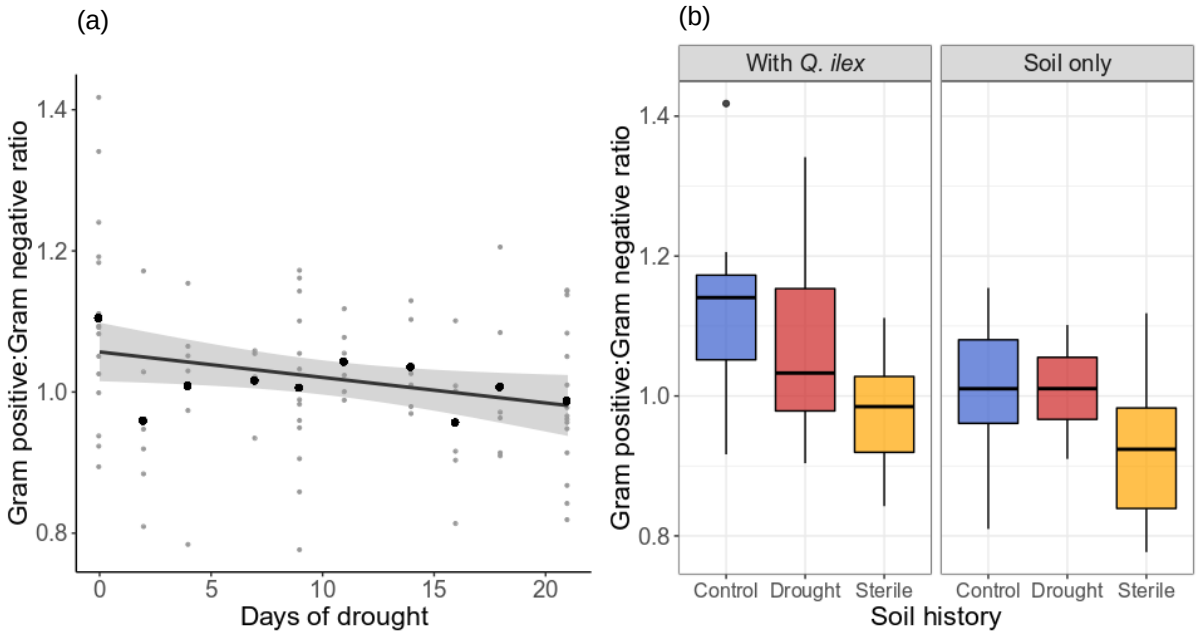

Fig. S6. Relationships between the ratio of Gram positive to Gram negative bacteria and (a) drought duration (days) ($P < 0.05$) and (b) soil history ($P < 0.001$) and the presence of *Q. ilex* ($P < 0.001$). For plot (a) small grey points represent individual samples and larger black points are mean values. For plot (b) soil treatments are 'Control', 'Drought' (pre-droughted) and 'Sterile' (pre-sterilised).

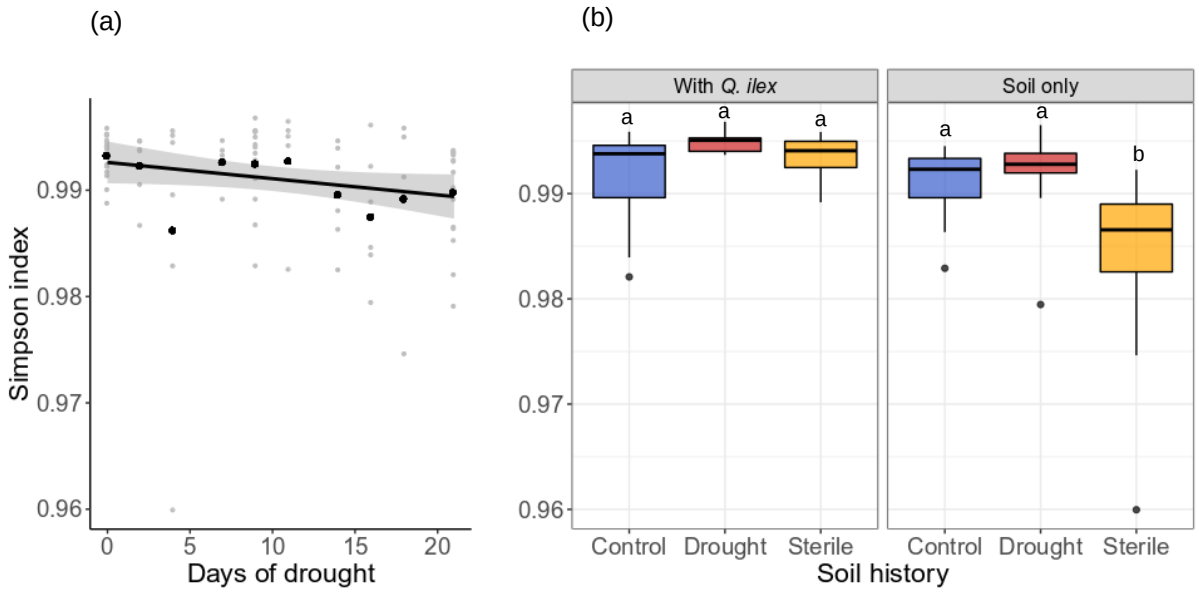

Fig. S7. Relationships between bacterial Simpson index and (a) drought duration (days) (*P* < 0.05) and (b) soil history and the presence of *Q. ilex* (significant interaction, *P* < 0.001). Soil treatments are 'Control', 'Drought' (pre-droughted) and 'Sterile' (pre-sterilised). For plot (a) small grey points represent individual samples and larger black points are mean values.

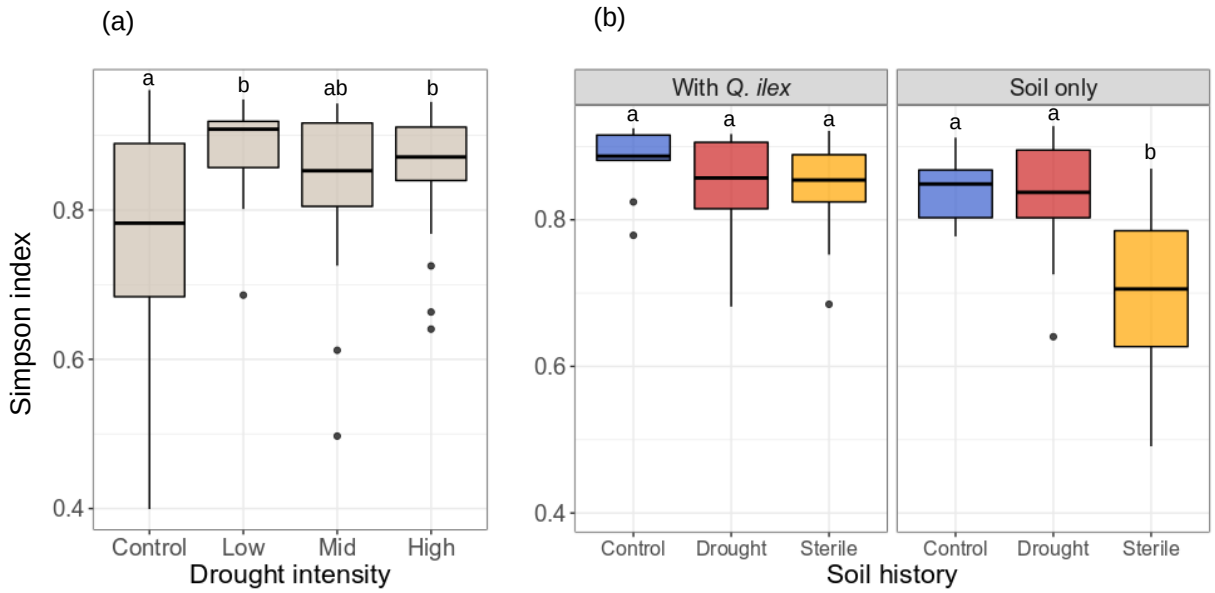

Fig S8. Relationships between fungal Simpson index and (a) drought duration as a categorical variable (Control, 0 days of drought; Low, 2-7 days; Mid, 9-14 days; High, 16-21 days; P < 0.001) and (b) soil history and the presence of *Q. ilex* (significant interaction, P < 0.01). Letters a and b denote treatments which differ from each other (Tukey HSD test, P < 0.05). Soil treatments are 'Control', 'Drought' (pre-droughted) and 'Sterile' (pre-sterilised).