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

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19 **Abstract**

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

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

41         Our results suggest that soil communities will not necessarily be able to maintain the  
42 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

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

93 Both soil microbial communities and droughts are highly variable, in both natural and  
94 experimental environments. The impact of water stress on plants and soils can depend on the  
95 timing or duration of the event or treatment (Hoover and Rogers, 2016; Mengtian et al.,  
96 2018), the proportional change in water availability, and the historical precipitation regime  
97 (Evans and Wallenstein, 2012; Bouskill et al., 2013). On this last point, soils that have  
98 experienced drought events may demonstrate ‘legacy effects’ whereby the soil community  
99 continues to show the impact of the drought for many years, and it may even modify the  
100 response to a later drought (de Vries et al., 2012; Bouskill et al., 2013; Kaisermann et al.,  
101 2017; Meisner et al., 2018). The impact of drought may also not elicit a linear response  
102 (Knapp et al., 2017), meaning that any negative effect may decrease or increase as the  
103 drought progresses, indicating acclimation or a ‘tipping point’, respectively. All this  
104 variability adds to the difficulty of drawing conclusions about the impacts of drought on soil  
105 microbes, and experiments are needed to test a more complex range of drought scenarios,  
106 including multiple levels of drought intensity in space and time and investigating if and when  
107 biologically relevant thresholds are exceeded (Beier et al., 2012).

108 The main objective of this study was to investigate the effect of increasing drought  
109 intensity on the bacterial and fungal communities of a Mediterranean soil. We focused on a  
110 holm oak (*Quercus ilex* L.) forest system, which is a predominant habitat throughout the  
111 Mediterranean Basin where greater drought frequency and severity is predicted (Field et al.,  
112 2014; Touma et al., 2015). We set up a greenhouse experiment with ten levels of drought and  
113 then used DNA-based amplicon sequencing and lipid analyses to observe the effects on soil  
114 bacterial and fungal communities in pots containing *Q. ilex* saplings. This experiment  
115 allowed us to determine whether bacterial communities were more responsive than fungal

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

123

## 124 **2. Materials and methods**

### 125 **2.1. Plant and soil material**

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

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

152

## 153 **2.2. Experimental design**

154 The drought treatment consisted of ten levels of drought, applied by withholding water for 0,  
155 2, 4, 7, 9, 11, 14, 16, 18, and 21 days. Each drought level therefore had 18 pots, divided into  
156 six replicate blocks (Supplementary Material Fig. S1). Soil samples were collected at the end  
157 of the drought period specific for each drought level. For pots with zero days of drought this  
158 sampling occurred at the end of the six week acclimation period. A subset of 90 of these soil  
159 samples were used for the DNA sequencing analysis, representing all drought levels and all  
160 soil history and plant treatments. This included 13-15 replicates for each combination of soil  
161 history  $\times$  presence of *Q. ilex*. One sample was discarded for the bacteria and two for the fungi  
162 (see Supplementary Material, Table S1 for full details of replication). Air temperature was  
163 monitored throughout the experiment using an EL-USB-2 data logger (Lascar Electronics,  
164 Wiltshire, UK) and had a mean of 26.7 °C. Soil temperature averaged 27.0 °C across the



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

172

### 173 **2.3. DNA library preparation and sequencing**

174 Total community DNA was extracted from approximately 0.25 g of soil using a PowerSoil  
175 DNA Isolation Kit following the manufacturer's protocol (MoBio, Carlsbad, USA). The  
176 hypervariable V3-V4 regions of the bacterial 16S rRNA gene was amplified using the 341F –  
177 806R primer pair (Klindworth et al., 2013) modified to include Illumina adapter sequences.  
178 Each 25 µl reaction mixture contained 1.5 µl of undiluted DNA extract, 1 µl each of the  
179 forward and reverse primers (10 µM), and 12.5 µl of Phusion High Fidelity PCR Master Mix  
180 with HF Buffer (ThermoFisher Scientific, Waltham, USA). Initial DNA concentration ranged  
181 between 3.9-10.2 ng/µl and DNA concentration was not standardized prior to PCR. PCR  
182 conditions were: initial denaturation at 98 °C for 30 s, followed by 30 cycles of denaturation  
183 at 98 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final  
184 extension at 72 °C for 4 min. After confirming successful amplification by agarose gel  
185 electrophoresis, PCR products were purified and normalized using Sequalprep plates  
186 (Thermofisher, USA), and subject to a second indexing PCR such that each sample received a  
187 unique combination of 6-nucleotide barcoded forward and reverse primers. The reaction  
188 mixture was as above, and the PCR program was an initial step at 95°C for 30 s, 8 cycles of:  
189 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

190 were again purified and normalized with Sequalprep plates and pooled for sequencing. The  
191 fungal ITS1 region was amplified in a one step approach using the primers ITS1f and ITS2  
192 augmented with multiplexing barcodes (Smith and Peay, 2014). Each reaction mixture  
193 contained 1 µl of the DNA extract, 1 µl of forward and reverse primers (10 µM), 200 µM  
194 dNTP's, 1X GC buffer and 0.4 U of Phusion DNA polymerase. PCR conditions were: initial  
195 denaturation at 98 °C for 30 s, followed by 40 cycles of 98 °C for 30 s, 55 °C for 30 s, and 72  
196 °C for 30 s, and a final extension at 72 °C for 10 min. Samples that failed to produce a PCR  
197 product were discarded, and PCR was repeated. Some fungal samples still failed to produce  
198 usable PCR products and were excluded from further analyses, but this only represented three  
199 samples out of 88 in total. Fungal PCR products were also purified and normalized with  
200 Sequalprep plates, and additionally extracted from a 1.5 agarose gel for size selection  
201 (approximately 200-500 bp which covers the entire range of length variation in the fungal  
202 ITS1 region) and to remove primer dimers. Then they were additionally purified using a  
203 QIAquick Gel Extraction Kit (Qiagen, Venlo, The Netherlands). Both fungal and bacterial  
204 libraries were quantified with real-time PCR using KAPA Library Quantification Kits (Kapa  
205 Biosystems, Wilmington, USA) to determine dilution factors for the sequencing protocol.

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

212

213

214

## 215 **2.4. Quality filtering and bioinformatic analysis**

216 The initial bioinformatic analysis of bacterial sequences used USEARCH software (Edgar,  
217 2013). Merging of paired-end reads was attempted, but low quality basecalls in the tail of  
218 both read directions precluded satisfactory merging. We therefore proceeded with separate  
219 parallel analyses of the forward and reverse reads (after truncating to 180bp and 150 bp  
220 respectively). Both reads gave qualitatively very similar results, so we focus hereafter on the  
221 longer forward read data. After primer removal sequences were filtered by quality leaving a  
222 total of 2.4 M high-quality sequences. Replicate and singleton sequences were removed, and  
223 a set of representative sequences of operational taxonomic units (OTUs) (97% similarity) was  
224 constructed using the UPARSE-OTU algorithm (Edgar, 2013). Chimeras were removed  
225 (leaving 11890 non-chimeric OTUs), and all original reads were mapped to the non-chimeric  
226 OTUs using the USEARCH algorithm with global alignments at an identity threshold of 0.97,  
227 yielding an OTU table. All subsequent steps used QIIME (Caporaso et al., 2010b). OTUs  
228 were aligned using the PyNAST algorithm (Caporaso et al., 2010a) and the Green Genes  
229 database (release 13\_8, DeSantis et al., 2006) as a template alignment. A subsampled OTU  
230 table was created by randomly sampling the original OTU table to avoid artefacts associated  
231 with library size. Samples that contained fewer sequences than the requested depth (2480)  
232 were omitted from the output OTU tables. Each OTU was taxonomically identified based on  
233 the 97% Green Genes database (release 13\_8) using the RDP classifier (Wang et al., 2007).

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

240 clustered to 97% similarity. Chimeras were filtered de novo and through the UNITE database  
241 of ITS1 sequences implemented in UCHIME, leaving a total of 3,323 non-chimeric OTUs,  
242 after which original sequences were mapped against these OTUs at a similarity threshold of  
243 97% and assembled in an OTU table. Representative sequences for each OTU were aligned  
244 to all fungal representative species in the UNITE database (Kõljalg et al., 2005) (release date  
245 20.11.2016) using the BLAST algorithm with default settings. The resulting hits were  
246 assigned to taxa, selecting the hit with the lowest E-value, provided it had a minimum E-  
247 value of  $1 \times 10^{-36}$  and a minimum alignment length of 75 bp. OTUs were subsequently  
248 assigned to functional groups if a genus was provided for the highest hit, and if it matched  
249 one of the genera with known lifestyles provided by Tedersoo et al. (2014). When the genus  
250 level was unknown, lifestyle was assigned at the family level if >80% of the genera within  
251 that family (represented by more than three genera) had the same lifestyle. As for bacteria,  
252 the OTU table was downsampled, to 12,332 reads per sample.

253

## 254 **2.5. Analysis of phospholipid fatty acids**

255 We used the amounts of phospholipid fatty acids (PLFAs) to quantify microbial biomass and  
256 the ratio of fungal to bacterial biomass. PLFA extraction and identification followed  
257 Frostegård et al. (1993), using 1 g of freeze-dried soil from each replicate. The abundance of  
258 individual fatty acids was determined as nmol per g of dry soil, and standard nomenclature  
259 was used (Tunlid et al., 1989). Concentrations of each PLFA were calculated based on the  
260 19:0 internal standard concentrations. Selection of bacterial PLFAs follows the selection of  
261 fatty acids by Frostegård and Bååth (1996), of which i14:0, i15:0, a15:0, i16:0, 16:1 $\omega$ 7c,  
262 a17:0, i17:0, cy17:0, 18:1 $\omega$ 7, and cy19:0 were present in our samples. We calculated the sum  
263 of i14:0, i15:0, a15:0, i16:0, a17:0, and i17:0 as an indicator of Gram-positive bacteria.  
264 Gram-negative bacteria were identified by the PLFAs 16:1 $\omega$ 7c, cy17:0, 18:1 $\omega$ 7, and cy19:0

265 (Zelles, 1999). The fungi were identified by 18:2ω6 (Frostegård et al., 1993; Frostegård et al.,  
266 2011). The ratio of 18:2ω6 to total bacterial PLFAs was used to estimate the ratio of fungal to  
267 bacterial biomass in soils (Bardgett et al., 1996; Frostegård and Bååth, 1996).

268

## 269 **2.6. Statistical analyses**

270 All statistical analyses were carried out using R v3.4.1 (R Core Team, 2016). The data for the  
271 fungal and bacterial communities were log-transformed relative abundance of each OTU  
272 within a sample. Non-metric multidimensional scaling (NMDS) plots (using Bray-Curtis  
273 distance with the *metaMDS* function in *vegan*) were constructed to visualize the separation  
274 between treatments. A permutational multivariate analysis of variance (PERMANOVA) was  
275 used to assess the effects of drought, soil history, and presence of a plant on the microbial  
276 communities, using the *adonis2* function in the *vegan* package. Block was included as a  
277 random factor, and 3000 permutations were used. This used Bray-Curtis dissimilarity indices  
278 generated by the *vegdist* function (also from *vegan*). A pair-wise PERMANOVA analysis  
279 was used to test for significant differences between pairs of factor levels (Martinez Arbizu,  
280 2017). We also carried out an NMDS analysis and PERMANOVA with the pre-sterilised soil  
281 removed, to check that this sterilised soil was not driving all of the significant soil history  
282 effect, and of the non-droughted pots only to assess the differences in the soil communities  
283 under ambient water conditions.

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

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

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

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

312

313

314

### 315 3. Results

#### 316 3.1. Initial differences in the soil communities

317 The NMDS analysis suggested that soil history and the presence of the plant affected the  
318 composition of the bacterial community before the start of the drought treatment (after six  
319 weeks of acclimation) (Fig. 1a, Fig. 1b, Table 1; PERMANOVA, soil history effect,  $P <$   
320  $0.001$ , pseudo  $R^2 = 0.25$ , plant effect,  $P < 0.001$ , pseudo  $R^2 = 0.16$ ). Note that the soil effect  
321 was driven by the strong difference between the pre-sterilised soil and the control and pre-  
322 droughted soils (Pairwise PERMANOVA,  $P < 0.05$ ), with no significant difference between  
323 the latter two soils. The presence of *Q. ilex* did not affect the fungal community (Fig. 1c), but  
324 soil history did, with the pre-sterilised soil clearly separated from the control and pre-  
325 droughted soils (Fig. 1d; PERMANOVA, soil history effect,  $P < 0.001$ , pseudo  $R^2 = 0.51$ ).

326 The diversity of the bacterial community measured by the Shannon index was  
327 affected by an interaction between the soil history effect and the presence of the plant (Table  
328 1; significant interaction,  $\chi^2 = 29.5$ ,  $P < 0.001$ ) such that the pre-sterilised soil had lower  
329 diversity than the other soils, but only in the absence of the plant (Supplementary Material,  
330 Fig. S4a). The Simpson index showed a very similar pattern, again with a significant  
331 interaction between plant and soil history (Table 1; significant interaction,  $\chi^2 = 21.9$ ,  $P <$   
332  $0.001$ ), again with the lowest value for pre-sterilised soil without the plant (Supplementary  
333 Material, Fig. S5a).

334 Soil history, but not the plant, affected the diversity of the fungal community.  
335 Diversity of the fungal community was higher in the pre-droughted than the pre-sterilised soil  
336 (Table 1;  $\chi^2 = 11.9$ ,  $P < 0.01$ ), with the control soil intermediate, and the diversity was 14.5%  
337 higher in the pre-droughted than the control soil, but not significantly higher (Supplementary  
338 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;  $\chi^2 = 9.3$ ,  $P <$   
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 ( $\chi^2 = 10.1$ ,  $P < 0.01$ )  
345 and was also higher in pots containing only soil than those containing *Q. ilex* ( $\chi^2 = 34.2$ ,  $P <$   
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 ( $\chi^2 = 24.3$ ,  $P < 0.001$ ). 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 ( $\chi^2 = 5.5$ ,  $P <$   
353 0.05) and was affected by soil history ( $\chi^2 = 21.3$ ,  $P < 0.001$ ), being lower in the pre-sterilised  
354 soil and in soils without *Q. ilex* ( $\chi^2 = 11.5$ ,  $P < 0.001$ ) (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



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

375

### 376 **3.4. Diversity of the microbial community during the drought experiment**

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

384

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

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

395 For bacterial communities, all combinations of soil history and plant-presence showed  
396 a decline in Shannon diversity between the control (0 days drought) treatment and the most  
397 extreme (21 days) drought treatment (a negative  $\Delta H$ ). Overall, for bacteria, a *t*-test on H in  
398 the control and the extreme drought treatments showed that the difference between Shannon  
399 diversity in the control and most extreme drought treatment was not significantly different  
400 (Table 4;  $t = 1.72$ ,  $P = 0.10$ ). In contrast, fungal Shannon diversity tended to increase between  
401 the most extreme drought treatment and the control drought level (a positive  $\Delta H$ ), by up to  
402 35% in one case, although in the case of soil with a history of previous drought and with  
403 presence of *Q. ilex* Shannon diversity decreased slightly. Overall, for fungi, a *t*-test did not  
404 reveal a significant difference between the two groups (control – 0 days drought – and most  
405 extreme drought – 21 days drought) ( $t = -1.88$ ,  $P$ -value = 0.07).

406

### 407 **3.5. Microbial taxonomic composition**

408 Overall, the most abundant bacterial phyla were *Proteobacteria* (36.0% of amplicon reads),  
409 *Actinobacteria* (18.5%), *Bacteroidetes* (13.4%), and *Verrucomicrobia* (6.4%). Other phyla  
410 that comprised a substantial (>2%) amount of the bacterial community were *Planctomycetes*  
411 (5.0%), *Chloroflexi* (4.8%), *Acidobacteria* (4.5%), and *Firmicutes* (2.9%). Drought affected

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

418 Twelve bacterial classes were present at >2%: *Alphaproteobacteria* (17.5%),  
419 *Actinobacteria* (11.4%), *Betaproteobacteria* (6.9%), *Gammaproteobacteria* (6.4%),  
420 *Saprosirae* (5.6%), *Thermoleophilia* (5.5%), *Cytophagia* (5.3%), *Deltaproteobacteria*  
421 (5.1%), *Planctomycetia* (3.3%), *Opitutae* (3.0%), *Bacilli* (2.8%), and *Anaerolineae* (2.6%).  
422 Again, the drought treatment affected most classes, but the effect was highly variable and  
423 depended on the presence of *Q. ilex* and soil history (Table S6). A large proportion of the  
424 fungal OTUs (45.8%) were unidentified, but filamentous saprotrophic fungi were the most  
425 abundant functional group (48.3%), followed by plant pathogens (2.5%) and saprotrophic  
426 yeasts (1.9%) (Table S7). Other functional groups at lower proportions (< 1.5%) of the total  
427 abundance included mycoparasites, ectomycorrhizal fungi, and saprotrophic white-rot fungi.

428

#### 429 **4. Discussion**

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

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

439

#### 440 **4.1. Differences in the soil communities without drought**

441 Our analysis of pots in the zero days of drought (control) treatment, which were sampled after  
442 six weeks of acclimation in the greenhouse, revealed that *Quercus ilex* plants impacted  
443 bacterial community composition and had a positive effect on bacterial diversity but did not  
444 affect the composition or diversity of the fungal community. This result may indicate either a  
445 larger influence of the rhizosphere (e.g. root exudation) on bacteria than fungi, or that fungi  
446 community composition responds more slowly due to their longer generation times (Rousk  
447 and Bååth, 2011). Consistent with this supposed relative inertia of fungal communities,  
448 previous soil history was found to have had a strong influence on the fungal community,  
449 which may have masked any effect of the plant. Indeed, soil history clearly separated both the  
450 bacterial and fungal communities in the sterilised soil from those in the control and pre-  
451 droughted soils. This showed that the sterilisation treatment successfully removed most of the  
452 bacteria and fungi present in the soil, and that the new microbial communities that colonised  
453 the soil were different than the original ones. Soil history also affected the diversity of  
454 bacteria and fungi in pots with zero days of drought. Bacterial community diversity was  
455 higher in pre-droughted and control soils compared with sterilised soil, but only in the  
456 absence of *Q. ilex*, indicating that the presence of a plant can quickly restore bacterial  
457 diversity. This is supported by previous studies that have shown the importance of plants for  
458 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).

482 Drought duration had a significant effect on the fungal:bacterial ratio, which increased  
483 with increasing drought. This result is broadly consistent with the majority of previous  
484 studies on this topic (Bapiri et al., 2010; Barnard et al., 2015). It is likely attributable to the  
485 chitinous cell walls of fungi, which should increase their resistance to environmental  
486 fluctuations, such as water stress (Holland and Coleman, 1987), and fungal hyphal growth  
487 (which most bacteria do not have) allowing them to cross small areas of dry soil (Yuste et al.,  
488 2011). Trends in fungal versus bacterial dominance, however, are variable and may depend  
489 on the trait measured (e.g. biomass or growth) and the method used (Strickland and Rousk,  
490 2010). Drought also decreased the ratio of Gram-positive:Gram-negative bacteria, which was  
491 surprising because Gram-positive bacteria are typically more drought resistant (Schimel et  
492 al., 2007). This trait, however, may be linked to the increase in root exudation by *Q. ilex*  
493 during increasing drought (Preece et al., 2018), because Gram-negative bacteria preferentially  
494 consume this type of labile carbon source, whereas Gram-positive bacteria tend to consume  
495 more recalcitrant C sources (Balasooriya et al., 2014; Naylor and Coleman-Derr, 2017).

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

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

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

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

523

#### 524 **4.3. Impact of drought on microbial-community diversity**

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

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

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

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



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

561

#### 562 **4.4. Are bacteria more affected than fungi by drought?**

563 Drought tended to affect the community composition of bacteria more than fungi, and the  
564 proportion of bacterial biomass compared with fungal biomass decreased under water stress,  
565 even though total microbial biomass was unaffected by drought. Taken together, this could  
566 suggest important changes in the future functioning of the bacterial community in soils  
567 exposed to water stress, for example relating to carbon and nutrient cycling (Schimel et al.,  
568 2007; Frank et al., 2015). During drought (in water-limited areas) decomposition rates slow,  
569 leading to a build up in soil organic matter (SOM) and lower N mineralisation (Borken and  
570 Matzner, 2009; Larsen et al., 2011; van der Molen et al., 2011; Sanaullah et al., 2012;  
571 Nguyen et al., 2018). The lack of strong effects of drought on fungal-community composition  
572 may be due to different responses being found depending on the strength of the drought, thus  
573 a lack of a clear unidirectional pattern: fungal diversity increased under low- and high-level  
574 drought but not mid-level drought. Soil bacteria are more abundant than fungi, so fungi may  
575 tend to take advantage of gaps where and when they can, resulting in a less standardised and  
576 more idiosyncratic response to disturbance. For example, we could speculate that under low  
577 drought there is some release of competition with bacteria, but during mid-level drought this  
578 is offset by inhibitive effects, such as low substrate diffusion rates and energetically  
579 expensive solute accumulation (Schimel et al., 2007). During extreme drought, some fungi

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

592

#### 593 **4.5. Does the presence of *Q. ilex* lessen the impacts of drought on microbial** 594 **communities?**

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

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

614 Water is so limiting to microbial growth (Manzoni et al., 2011) that any positive  
615 effect of the plant under drought conditions of more than a few days was likely minimal.  
616 Although without measurements of water potential this is hard to confirm. An increase in  
617 carbon inputs to the soil by plants also likely only affects a very isolated area near the roots  
618 (Dennis et al., 2010), and these inputs cannot easily diffuse in dry soil to be able to influence  
619 a large proportion of the soil microbes. An additional consideration is that the length of this  
620 experiment may not have been sufficient for demonstrating the full impacts on fungi, which  
621 have relatively slow growth compared to bacteria. For example, bacterial turnover time tends  
622 to be days to weeks, whereas fungal turnover is usually weeks to months (Rousk and Bååth,  
623 2011). The complexity of the microbial community may also mask some impacts that affect  
624 some groups of the community. Many specific bacterial and fungal taxa showed an  
625 interaction between the drought treatment and the presence of *Q. ilex*, although the direction  
626 of the responses was not consistent. For example, *Gammaproteobacteria* relative abundance  
627 was negatively correlated with increasing drought when *Q. ilex* was absent, but there was no

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

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

641

#### 642 **4.6. Are soil microbial communities with a history of drought more resistant to** 643 **drought?**

644 Recent studies have identified ‘legacy effects’ of drought-stressed soils. This means that for  
645 soils that have been previously water stressed, a subsequent drought may have a stronger or  
646 weaker impact on the soil community than a soil without such a history (de Vries et al., 2012;  
647 Evans and Wallenstein, 2012; Bouskill et al., 2013; Hawkes and Keitt, 2015; Kaisermann et  
648 al., 2017; Meisner et al., 2018). A stronger impact could be due to the loss of resistance or  
649 resilience of a repeatedly disturbed soil, whereas a weaker impact would imply the selection  
650 of taxa that are better adapted to the conditions. We found no interaction between drought  
651 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

#### 701 **4.7. The future of soil communities under drought**

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

717

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731 **References**

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Treatment factor	Bacteria						Fungi					
	Composition		Shannon diversity		Simpson index		Composition		Shannon diversity		Simpson index	
	$R^2$	$P$	$\chi^2$	$P$	$\chi^2$	$P$	$R^2$	$P$	$\chi^2$	$P$	$\chi^2$	$P$
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	NS	NS	29.5	***	21.9	***	NS	NS	NS	NS	9.3	**

\* Plant

1027 **Table 1. Factors affecting initial differences in soil community composition (results of PERMANOVA using the *adonis2* function in the R *vegan***  
1028 **package) and Shannon diversity (results of linear mixed effects model). Asterisks represent the  $P$ -value: \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ .**  
1029 **NS = non-significant.**

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1031

Treatment factor	Bacteria				Fungi			
	Continuous		Factor		Continuous		Factor	
Type of drought variable	$R^2$	$P$	$R^2$	$P$	$R^2$	$P$	$R^2$	$P$
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 \*\*\* =  $P < 0.001$ .**

Factor	Bacteria				Fungi			
	Shannon		Simpson		Shannon		Simpson	
	$\chi^2$	$P$	$\chi^2$	$P$	$\chi^2$	$P$	$\chi^2$	$P$
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	<i>Q. ilex</i>	$\Delta H$	
		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.  $\Delta H$  (% change after most intense drought compared to control) of the Shannon diversity (H) of the bacterial and fungal communities to drought.**

Fig. 1

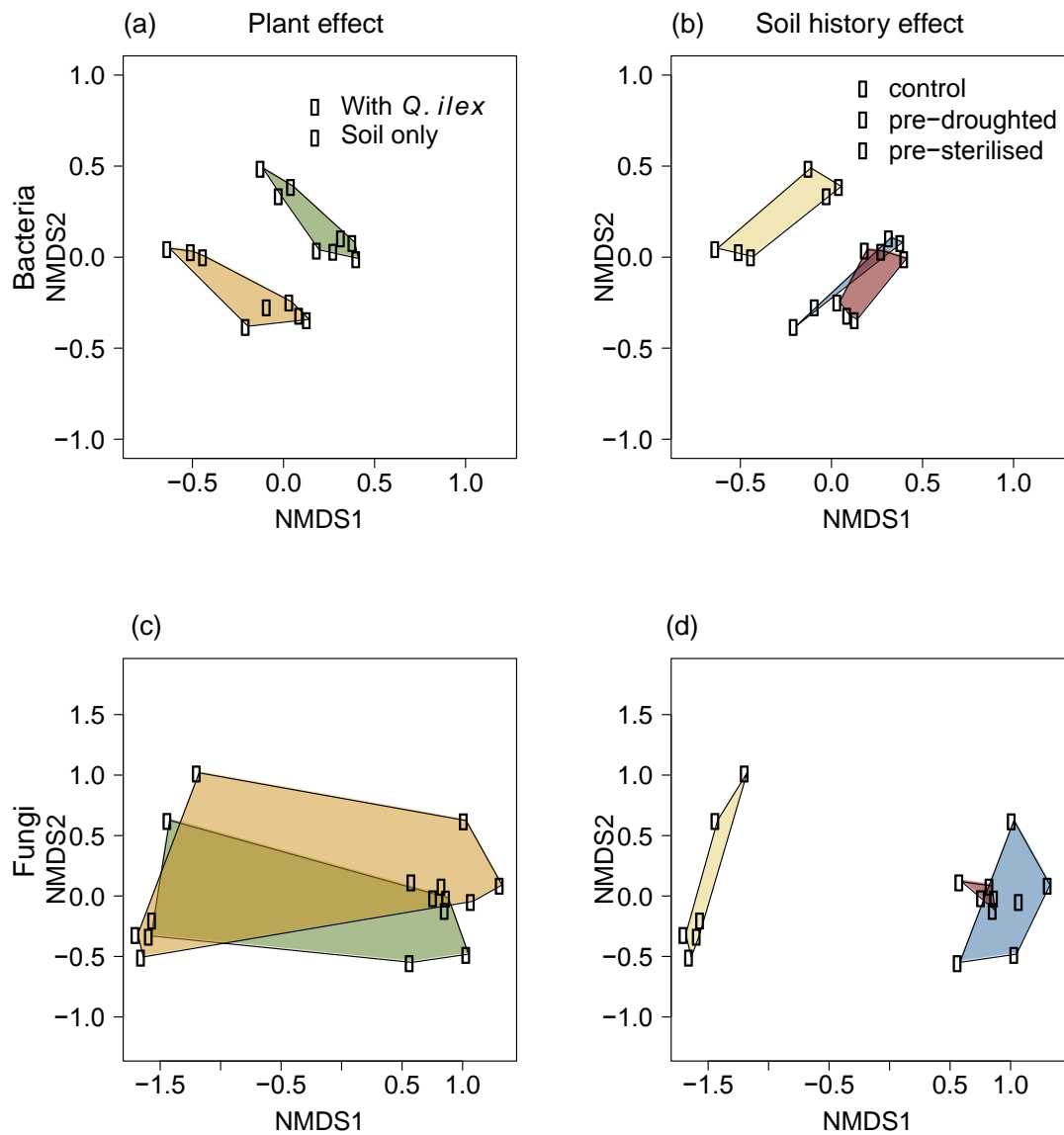


Fig. 1. NMDS plots based on Bray-Curtis dissimilarities of the (a,b) bacterial and (c,d) fungal communities prior to onset of drought (drought duration of zero days), showing the difference due to the presence of *Q. ilex* and the soil history. Points represent individual samples, convex hulls encompass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



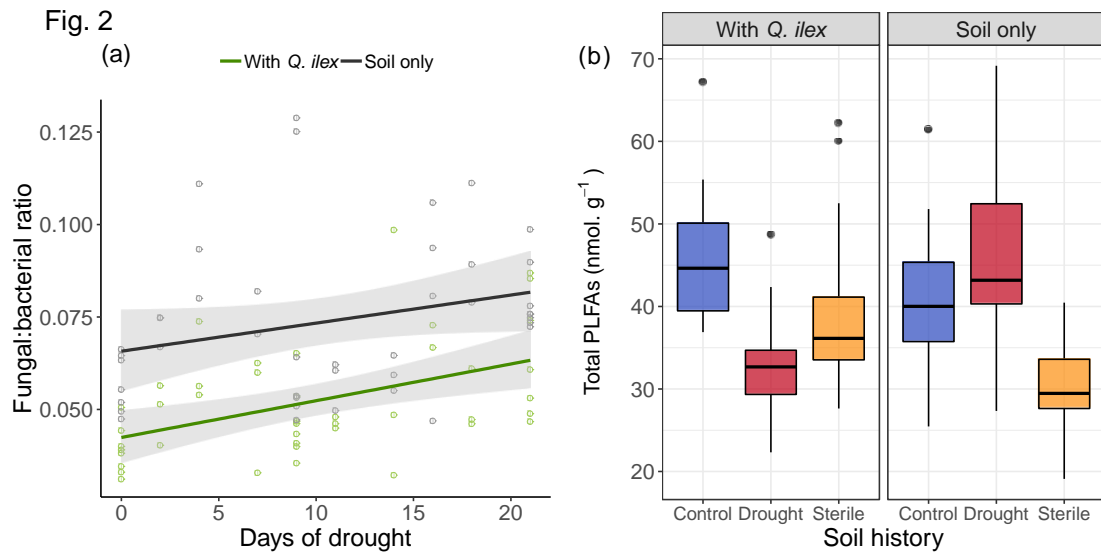


Fig. 2. (a) Fungal:bacterial ratio for the drought durations with *Q. ilex* present (green lines and dots) and for soil only (black lines and grey dots) There was a positive relationship with drought ( $P < 0.01$ ) and a positive effect of plant-presence ( $P < 0.001$ ). (b) Total PLFAs (nmol g<sup>-1</sup>) 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

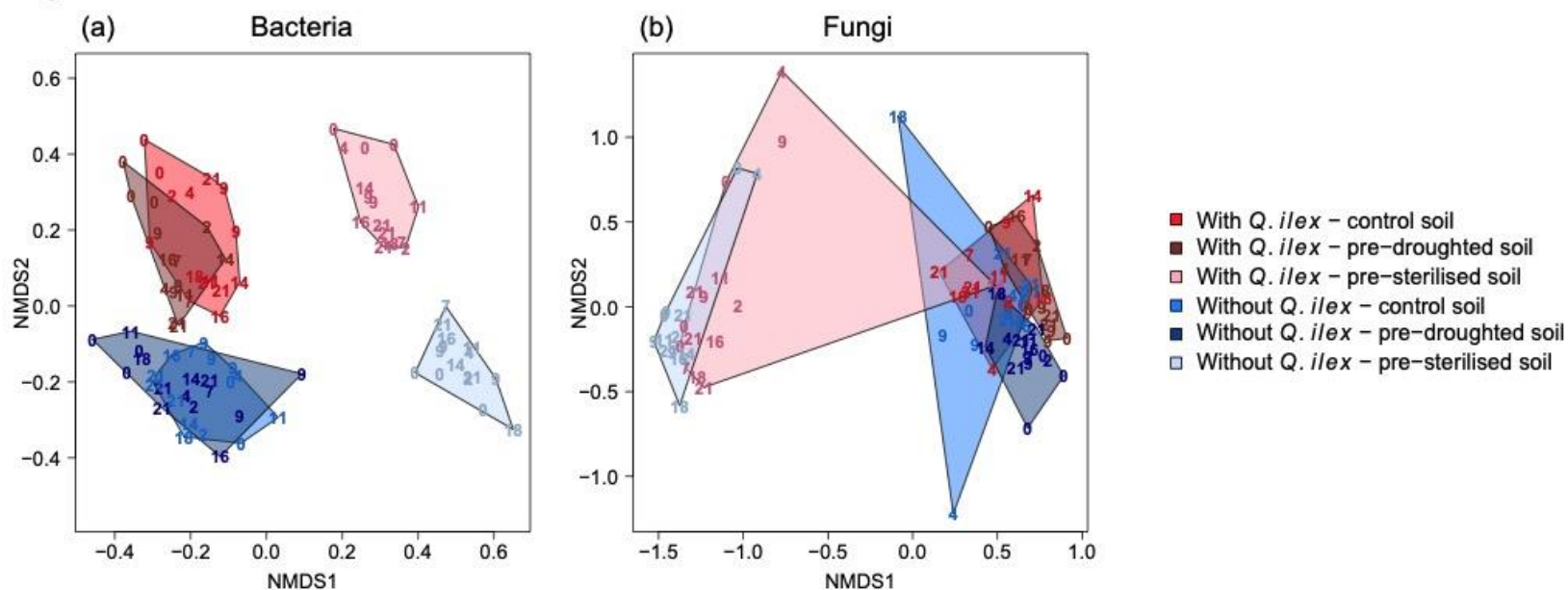


Fig. 3. NMDS plots based on Bray-Curtis dissimilarities of the (a) bacterial and (b) fungal communities after drought treatments of 0 to 21 days. Numbers (0 to 21) show the length in days of the drought treatment, and represent individual samples. The six combinations of soil history and plant presence 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.

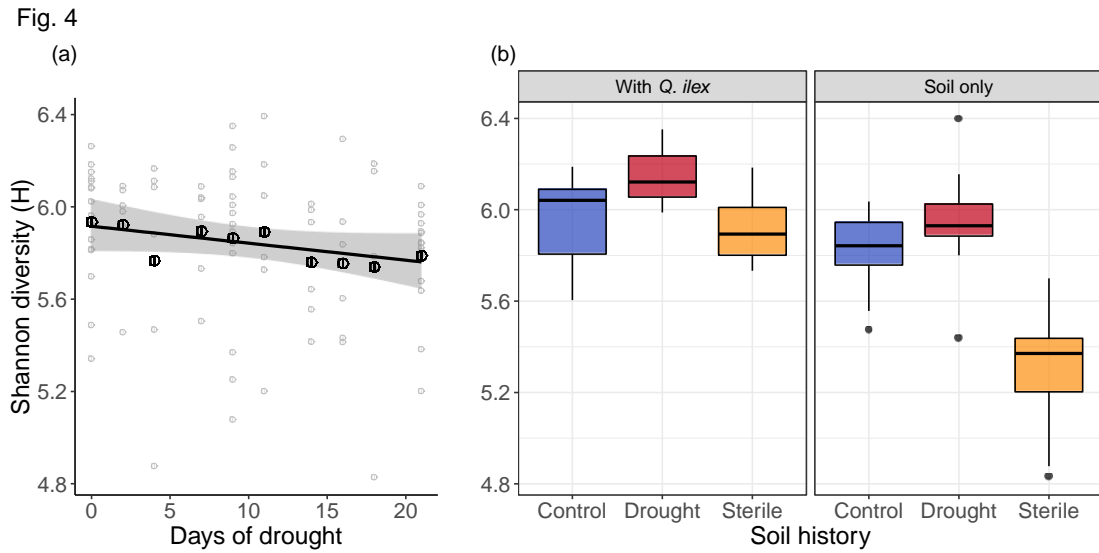


Fig. 4. Relationships between bacterial Shannon diversity and (a) drought duration (days) ( $P < 0.001$ ) and (b) soil history ( $P < 0.01$ , all three soil histories differed from each other) and the presence of *Q. ilex* (significant interaction,  $P < 0.001$ ).  $n = 15$  for each plant and soil combination. 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

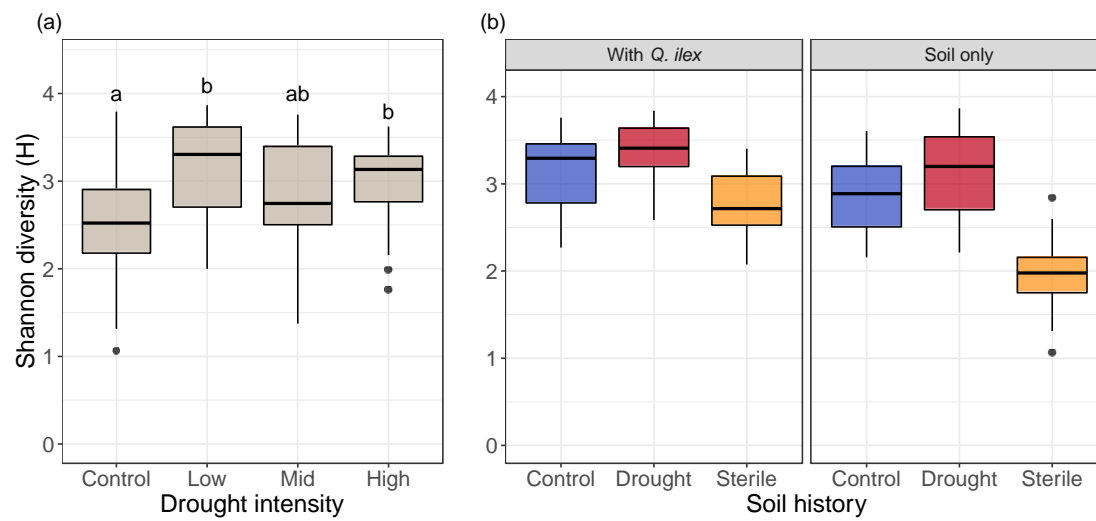


Fig. 5. Relationships between fungal Shannon diversity and (a) drought duration as a categorical variable (Control, 0 days of drought; Low, 2-7 days; Mid, 9-14 days; High, 16-21 days;  $P < 0.001$ ) and (b) soil history ( $P < 0.01$ , pre-sterilised soil differs from the control and pre-droughted soil) 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
	<b>n</b>	<b>n</b>	<b>n</b>	<b>n</b>	<b>n</b>	<b>n</b>
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			
	Continuous		Factor		Continuous		Factor	
Type of drought variable	$R^2$	$P$	$R^2$	$P$	$R^2$	$P$	$R^2$	$P$
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	<i>F</i>	<i>R</i> <sup>2</sup>	<i>P</i> adj.
Bacteria	D <sub>con</sub> – D <sub>low</sub>		1.65	0.049	0.198
	D <sub>con</sub> – D <sub>mid</sub>		2.14	0.049	0.018
	D <sub>con</sub> – D <sub>high</sub>		2.55	0.059	<b>0.006</b>
	D <sub>low</sub> – D <sub>mid</sub>		1.00	0.022	1.000
	D <sub>low</sub> – D <sub>high</sub>		1.49	0.033	0.366
	D <sub>mid</sub> – D <sub>high</sub>		2.04	0.037	0.060
Fungi	D <sub>con</sub> – D <sub>low</sub>		0.86	0.028	1.000
	D <sub>con</sub> – D <sub>mid</sub>		0.69	0.017	1.000
	D <sub>con</sub> – D <sub>high</sub>		0.93	0.022	1.000
	D <sub>low</sub> – D <sub>mid</sub>		0.96	0.023	1.000
	D <sub>low</sub> – D <sub>high</sub>		0.82	0.019	1.000
	D <sub>mid</sub> – D <sub>high</sub>		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<sub>con</sub> = control (0 days drought), D<sub>low</sub> = low (2-7 days drought), D<sub>mid</sub> = mid (9-14 days drought), D<sub>high</sub> = high (16-21 days drought).**

	Pairs	n	<i>F</i>	<i>R</i> <sup>2</sup>	<i>P</i> adj.
Bacteria	S <sub>con</sub> – S <sub>dro</sub>		2.93	0.049	<b>0.003</b>
	S <sub>con</sub> – S <sub>ste</sub>		11.76	0.169	<b>0.003</b>
	S <sub>dro</sub> – S <sub>ste</sub>		12.16	0.176	<b>0.003</b>
Fungi	S <sub>con</sub> – S <sub>dro</sub>		5.95	0.096	<b>0.003</b>
	S <sub>con</sub> – S <sub>ste</sub>		33.65	0.384	<b>0.003</b>
	S <sub>dro</sub> – S <sub>ste</sub>		40.76	0.421	<b>0.003</b>

**Table S4. Results of pairwise PERMANOVA of microbial composition (Bray-Curtis dissimilarity indices) between different soil histories. Soil histories are: S<sub>con</sub> = control, S<sub>dro</sub> = pre-droughted, S<sub>ste</sub> = pre-sterilised.**

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

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

**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.**



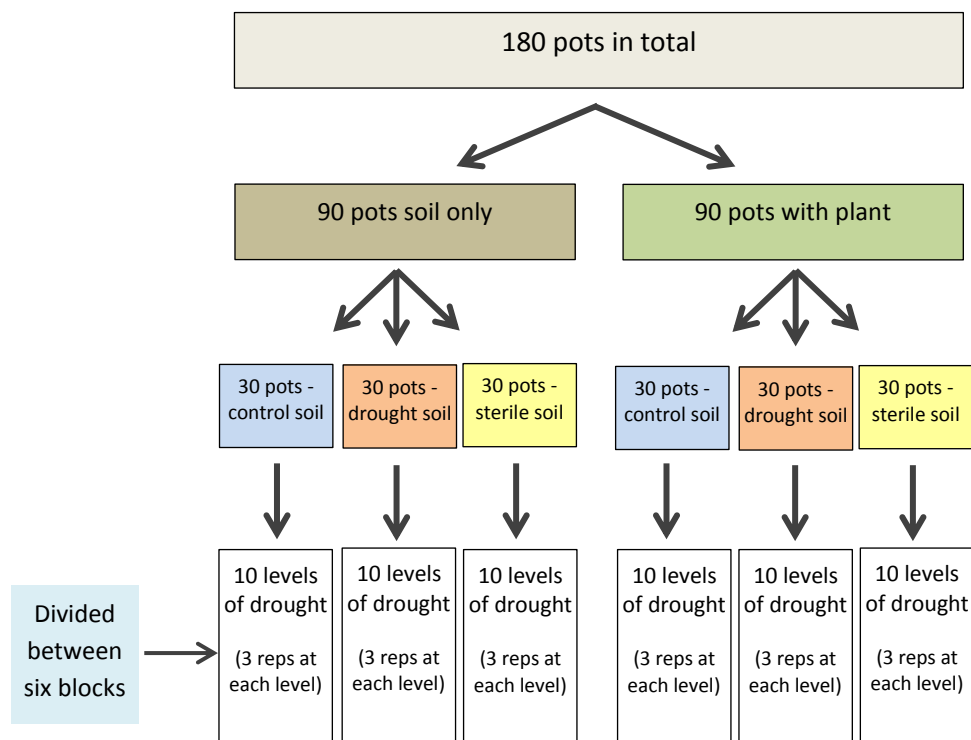
Class	Change in relative abundance					
	Drought * Soil history	Drought * Plant	Plant * Soil history	Drought effect	Soil history effect	Plant effect
<i>Alphaproteobacteria</i>	NS	NS	NS	NS	NS	NS
<i>Actinobacteria</i>	NS	NS	NS	+ve correlation	NS	soil only > with <i>Q. ilex</i>
<i>Betaproteobacteria</i>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

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

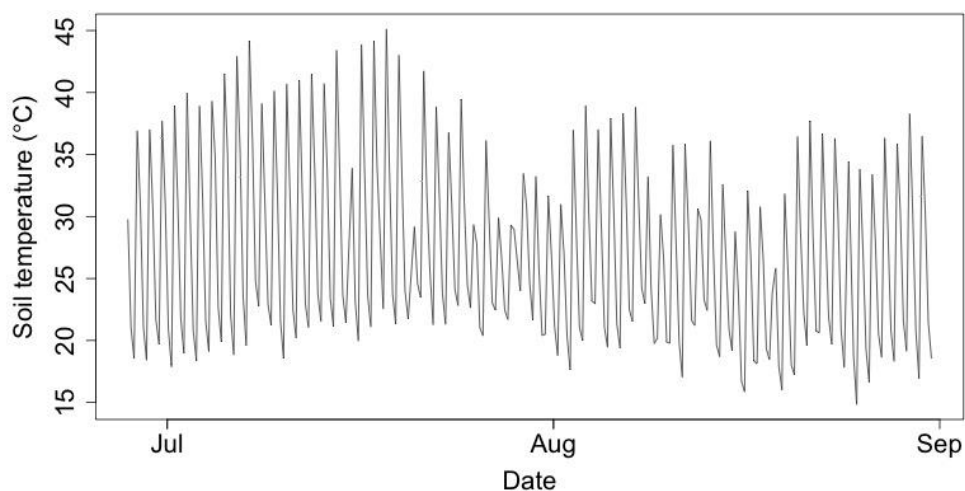
**Table S6. Summary of differences in relative abundances of bacterial classes contributing > 2% of total reads during the drought experiment. NS, not significant. +ve = positive and -ve = negative.**

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 <i>Q. ilex</i>
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 <i>Q. ilex</i>

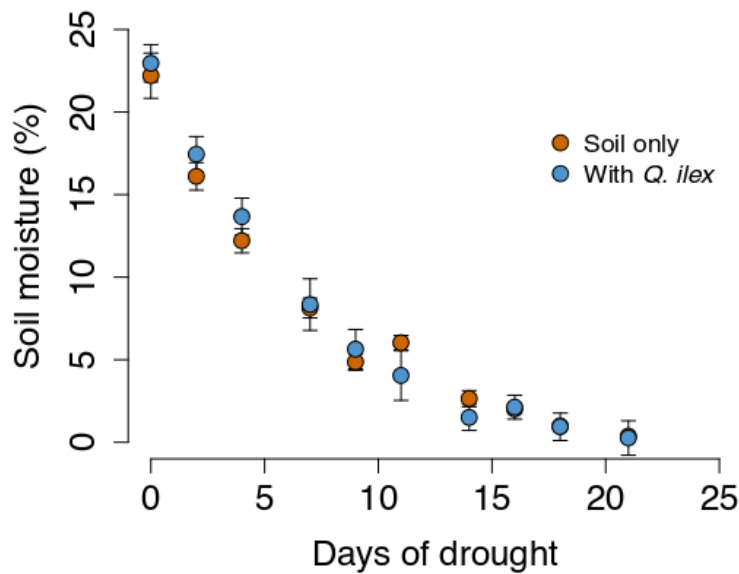
**Table S7. Summary of differences in relative abundance of fungal functional groups contributing > 1.5 % of total reads during the drought experiment. NS, not significant. +ve = positive and -ve = negative.**



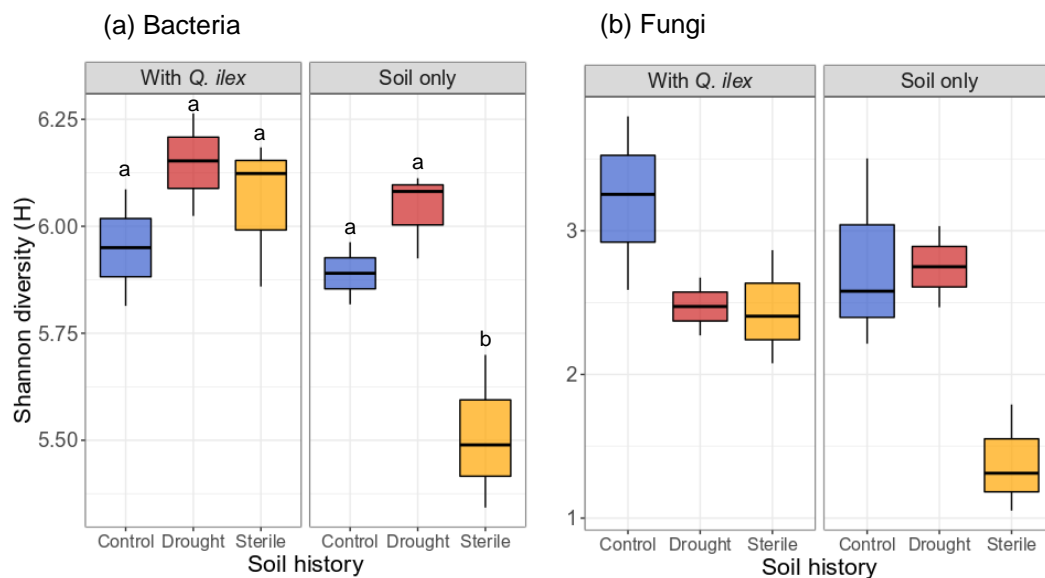
**Fig. S1. Outline of the original experimental design, showing number of replicates in each treatment combination. Note that while there were three replicates of each treatment combination (plant  $\times$  soil history  $\times$  drought level) there were six experimental blocks. This was due to a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



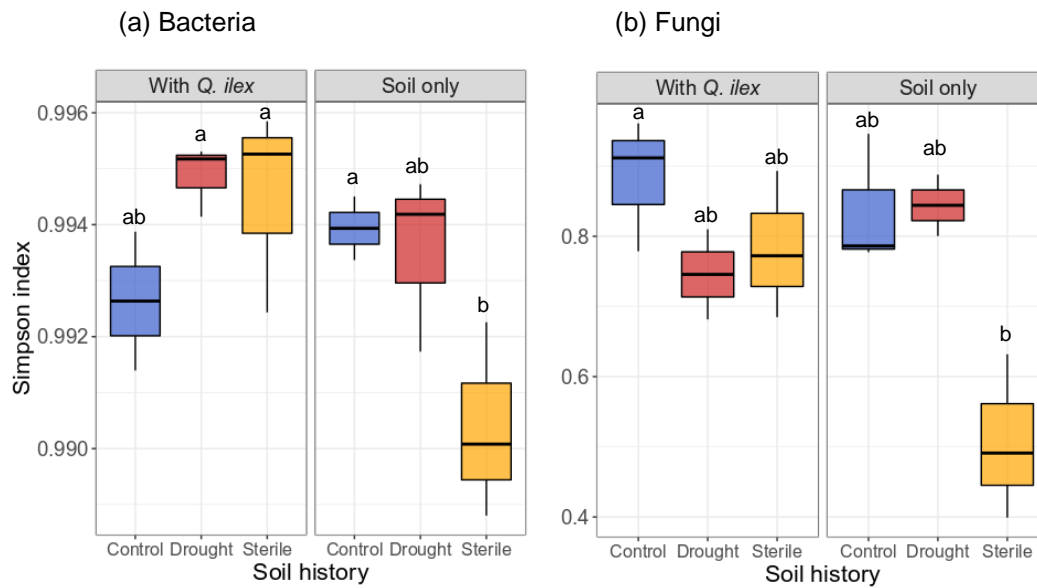
**Fig. S2. Mean soil temperature throughout the experiment measured in five pots (due to availability of soil moisture probes).**



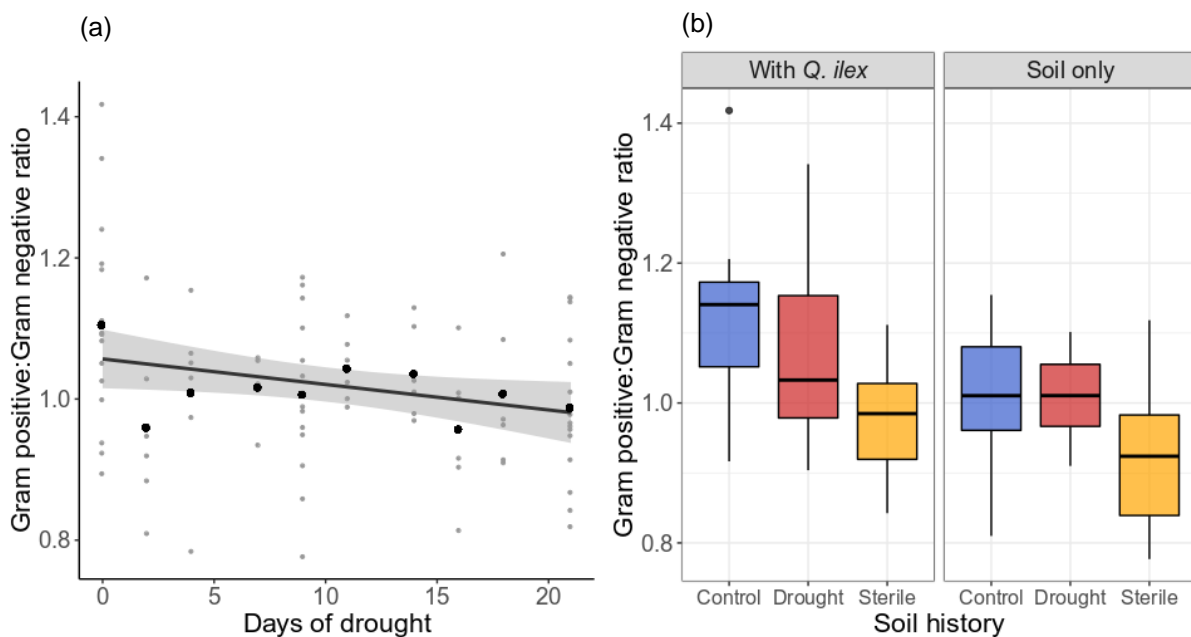
**Fig. S3.** Percentage soil moisture for the ten different drought levels measured at the end of each drought treatment. Points are means for each drought level, and shown in dark orange for pots without the plant ('soil only') and blue for pots with the plant ('with *Q. ilex*'). Standard error bars 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.



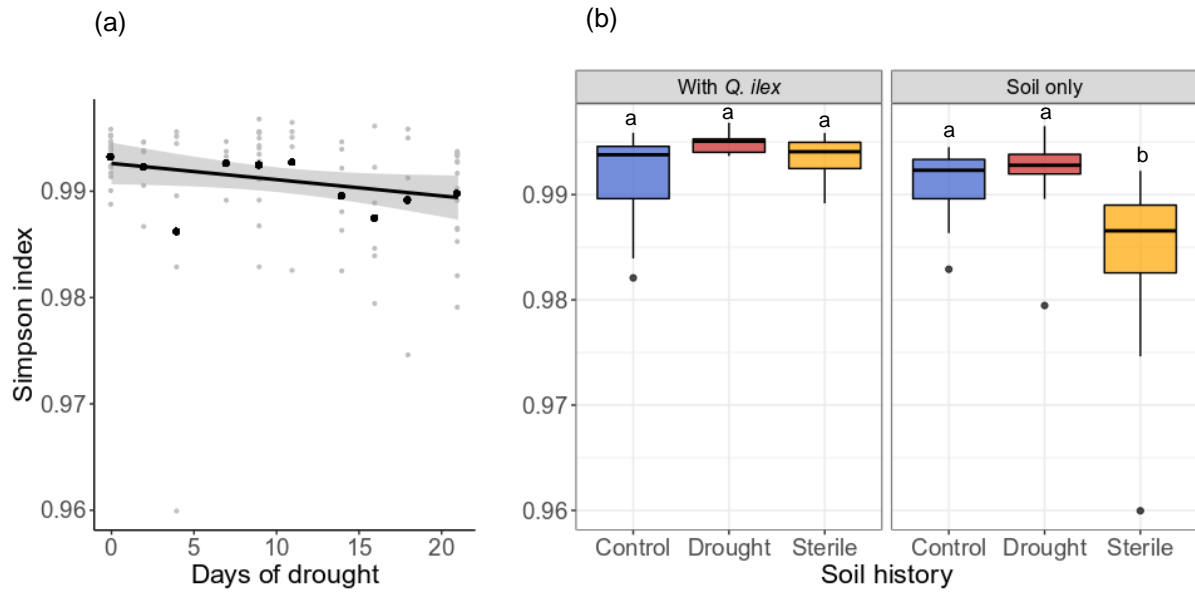
**Fig. S4.** Relationships between (a) bacterial and (b) fungal Shannon diversity and soil history and the presence of *Q. ilex*, prior to onset of drought (drought duration of zero days). Soil treatments are 'Control', 'Drought' (pre-droughted) and 'Sterile' (pre-sterilised). For bacteria there was 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).



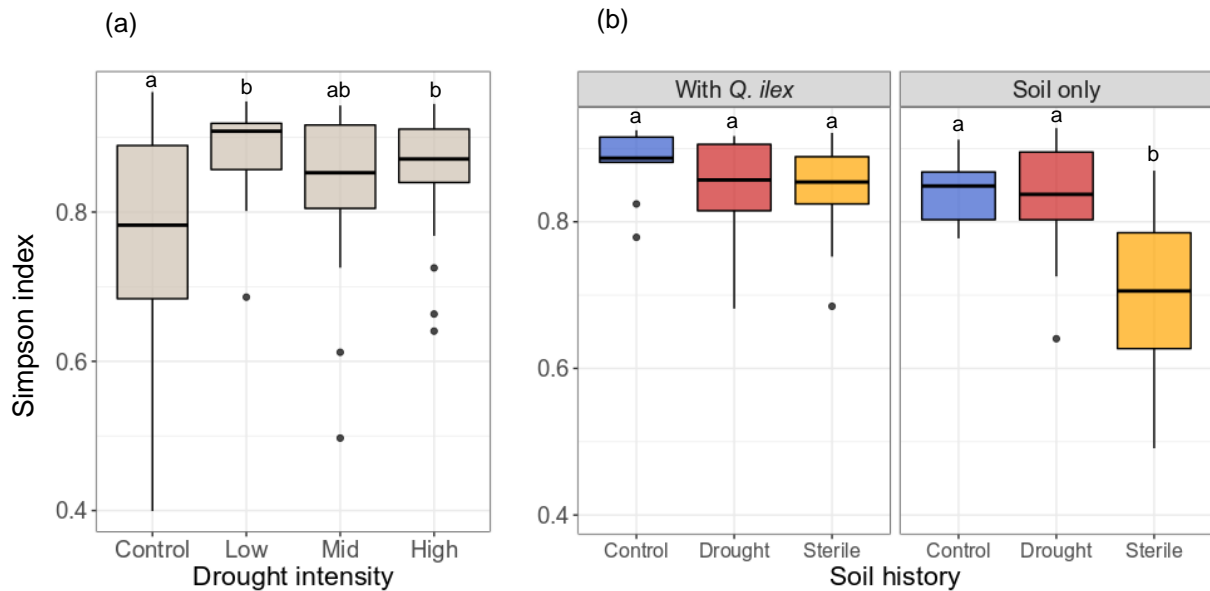
**Fig. S5. Relationships between (a) bacterial and (b) fungal Simpson index and soil history and the presence of *Q. ilex*, prior to onset of drought (drought duration of zero days). Soil treatments are 'Control', 'Drought' (pre-droughted) and 'Sterile' (pre-sterilised). Letters (a and b) signify significant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