Quantitative losses vs. qualitative stability of ectomycorrhizal community responses to 3 years of experimental summer drought in a beech-spruce forest

Running head: Ectomycorrhizal responses to extended drought

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Abstract

Forest ecosystems in central Europe are predicted to face an increasing frequency and severity of summer droughts because of global climate change. European beech and Norway spruce often coexist in these forests with mostly positive effects on their growth. However, their different below-ground responses to drought may lead to differences in ectomycorrhizal (ECM) fungal community composition and functions which we examined at the individual root and ecosystem levels. We installed retractable roofs over plots in Kranzberg Forest (11°39′42″E, 48°25′12″N; 490 m a.s.l.) to impose repeated summer drought conditions and assigned zones within each plot where trees neighboured the same or different species to study mixed species effects. We found that ECM fungal community composition changed and the numbers of vital mycorrhizae decreased for both tree species over 3 drought years (2014–2016), with the ECM fungal community diversity of beech exhibiting a faster and of spruce a stronger decline. Mixed stands had a positive effect on the ECM fungal community diversity of both tree species after the third drought year. Ectomycorrhizae with long rhizomorphs increased in both species under drought, indicating long-distance water transport. However, there was a progressive decline in the number of vital fine roots during the experiment, resulting in a strong reduction in enzyme activity per unit volume of soil. Hydrolytic enzyme activities of the surviving ectomycorrhizae were stable or stimulated upon drought, but there was a large decline in ECM fungal species with laccase activity, indicating a decreased potential to exploit nutrients bound to phenolic compounds. Thus, the ectomycorrhizae responded to repeated drought by maintaining or increasing their functionality at the individual root level, but were unable to compensate for quantitative losses at the ecosystem level. These findings demonstrate a strong below-ground impact of recurrent drought events in forests.

Introduction

European beech (*Fagus sylvatica* L.) and Norway spruce (*Picea abies* (L.) KARST.) have a wide ecological range and are among the dominant tree species in mesic temperate forest ecosystems across Europe (Ellenberg, 1988; Fang & Lechowicz, 2006). Together with close relatives, these ectomycorrhizal (ECM) tree species are major components of ecosystems throughout the Holarctic realm (Fang & Lechowicz, 2006; Lockwood et al., 2013). However, both species are at risk from the increased frequency and intensity of droughts that are predicted by future climate change scenarios (Geßler et al., 2007; Spiecker, 1995; Young et al., 2017), with spruce being particularly vulnerable because of its shallow roots system and its low drought tolerance (Boden, Kahle, von Wilpert, & Spiecker, 2014).

Both species form monospecific and mixed forest stands, with spruce mostly exhibiting increased overall productivity when growing in mixture (Pretzsch et al., 2014). These positive mixed stand effects have been attributed to improved soil properties and increased overall biodiversity by beech (Ammer, Bickel, & Kölling, 2008). In addition, below-ground resource partitioning is likely to contribute to the positive effects of mixture as beech shifts its fine roots from upper to lower soil depths when growing alongside spruce because of competition (Bolte & Villanueva, 2006; Goisser et al. 2016). Under severe summer drought conditions, spruce can adapt by decreasing its fine-root growth (Puhe, 2003) while maintaining its standing fine-root biomass (Nikolova, Andersen, Blaschke, Matyssek, & Häberle, 2010), whereas beech exhibited slightly increased fine-root growth during the severe summer drought of 2003 (Nikolova et al., 2010). Thus, water limitation evokes different below-ground responses in these species (Schume, Jost, & Hager, 2004), with spruce decreasing water consumption and growth in the early stages of drought (Dobson, Taylor, & Freer-Smith, 1990; Maier-Maercker, 1998) and beech continuing

to grow (Burkhardt & Pariyar, 2016). The distinct physiological responses of these tree species to drought suggest that their ectomycorrhizae will be exposed to different conditions under the same drought scenario.

From the thousands of ECM fungal species potentially forming ectomycorrhizae (Tedersoo, et al., 2014), at the plot level, fine roots of spruce and beech have been found to form ectomycorrhizae with an estimated 60 species of Basidiomycota and Ascomycota (Pena et al., 2010; Taylor, Martin, & Read, 2000). ECM fungi form a hyphal mantle around the primary roots and hyphal networks outside the roots (extramatrical mycelia), which constitute functional extensions of the plant roots (Finlay & Read, 1986). The ability of ECM fungi to exploit the nutrients and water contained in the surrounding soil gives them the potential to improve the nutritional status of trees associated with ECM fungi and to contribute to tree water uptake from the soil, attenuating drought stress in those trees (Allen, 2007; Lehto & Zwiazek, 2011). ECM fungi mediate plant nutrient uptake either directly in solubilised form or following enzymatic mobilisation from organic debris (Abuzinadah, Finlay, & Read, 1986; Pritsch & Garbaye, 2011). Extracellular enzyme activities (EAs) of ectomycorrhizae are considered functional traits that are indicative of changing conditions in plant–soil ecosystems (Koide, Fernandez, & Malcolm, 2014). Although nutrient turnover processes are generally decreased in dry soils (Sardans & Peñuelas, 2005), ECM fungi can overcome a local soil water deficit by transporting water through their mycelia and particularly through their rhizomorphs (Brownlee, Duddridge, Malibari, & Read, 1983; Duddridge, Malibari, & Read, 1980; Lilleskov, Bruns, Dawson, & Camacho, 2009), thereby retaining or even increasing the potential for nutrient mobilisation. Besides, a local soil water deficit can be attenuated by hydraulic lift (Caldwell et al., 1998): At night water moves passively through roots of deep rooting trees such as *Fagus sylvatica* from

89 deep soil layers (higher water potential) to shallow soil layers (lower water potential) where
90 nutrients and fine roots are abundant.

91 On the basis of the organisation of their extramatrical mycelia, ECM fungi can be categorised
92 as contact, short-distance, medium-distance or long-distance exploration types (Agerer, 2001).
93 ECM fungi of the long-distance and medium-distance exploration types form rhizomorphs,
94 increasing water transport to the roots (Cairney, 1992; Duddridge et al., 1980). Bakker, Augusto,
95 and Achat (2006) found that moist forest sites contained more contact types, while dry forest
96 sites contained more short-distance and long-distance types, indicating an increase in
97 functionality of ECM fine-root systems with respect to water transport. However, clear evidence
98 for exploration type preferences to local soil moisture conditions is still lacking as the locations
99 examined by Bakker et al. (2006) also differed in tree species, soil type and nutrient status (Lehto
100 & Zwiazek, 2011).

101 ECM fungi are exposed to periodic soil drought even in regions with normally adequate
102 amounts of precipitation. Drought tolerance differs among ECM fungal species (reviewed in
103 Lehto & Zwiazek, 2011) and probably also among populations of a species (Lamhamedi,
104 Bernier, & André-Fortin, 1992), resulting in diverse changes in ECM fungal community
105 composition under drought (Cavender-Bares, Izzo, Robinson, & Lovelock, 2009; di Pietro,
106 Churin, & Garbaye, 2007; Richard et al., 2011; Swaty, Deckert, Whitham, & Gehring, 2004). To
107 determine whether altered ECM fungal community composition is critical to ecosystem
108 functioning or is indicative of a plastic functional system with high adaptive potential, it is
109 important to also consider the functional traits of these communities, such as the capacity to
110 transport water through rhizomorphs or the activity of their extracellular enzymes. (Dahlberg,
111 2001; Kipfer, Wohlgemuth, van der Heijden, Ghazoul, & Egli, 2012). ECM fungal community

112 composition ultimately determines the functionality of a fine-root system through the different
113 properties of the ECM fungal species involved (Cairney, 1999; Godbold & Berntson, 1997; Shi,
114 Guttenberger, Kottke, & Hampp, 2002) and so alterations in community composition are likely
115 to alter the function of the fine-root system. If such alterations are driven by a certain stress
116 factor, they may affect functionality in the direction of stress resistance or resilience.

117 In the present study, we examined the responses of the ECM fungal communities of beech
118 and spruce under repeated summer drought as part of the Kranzberg Roof Experiment (KROOF)
119 project, which is a throughfall exclusion experiment being carried out in a maturing (age 60–70
120 years) beech–spruce forest [see Pretzsch et al. (2014) for a detailed description of the
121 experimental site].

122 We investigated how ECM fungal communities of beech and spruce reacted upon repeated
123 summer drought in terms of ECM fungal diversity and community composition, the potential to
124 transport water through ECM fungal rhizomorphs and the potential activity of extracellular
125 enzymes of vital ectomycorrhizae. We addressed three hypotheses: H1, repeated years of
126 throughfall exclusion influence ECM fungal community composition and functions more
127 strongly in spruce than in beech; H2, repeated drought leads to changes in the functionality of the
128 ECM fine-root system towards traits that are related to drought resistance, irrespective of the tree
129 species; and H3, the negative effects of drought on ECM fungal communities of beech and
130 spruce are attenuated in mixed stands compared with monospecific stands.

131 **Materials and methods**

132 *Site description and climatic conditions*
133 This study was conducted in Kranzberg Forest, which is a mixed mature forest situated in
134 southern Germany (11°39′42″E, 48°25′12″N; 490 m a.s.l.). The study site had an average annual

precipitation rate of 723 ± 27 mm year^{-1} between October 2011 and October 2016, of which approximately 500 mm fell during the growing season (April–October), and an annual mean temperature of 8.4 ± 0.4 °C, with an average of 13.1 ± 0.5 °C during the growing season (Fig. 1). Weather conditions at the site differed strongly during study years (2013–2016) including an extremely hot and dry period in 2015 [rainfall sum in summer (June to August) reduced by 56% compared to mean of rainfall sums in summers 2014 and 2016] imposing natural drought on trees also on control plots (Fig. 1). This site is dominated by European beech and Norway spruce, with an average age of 82 ± 4 years (beech) and 62 ± 2 years (spruce) in 2013. The soil is a nutrient-rich luvisol developed from loess over tertiary sediments [eutric cambisols; Food and Agriculture Organization (FAO) classification].

In 2010, 12 plots (100–200 m²) were established at the study site by digging trenches to a depth of approximately 1 m, where a water impermeable clay layer prevents water transport from below. Water impermeable canvas was then used to vertically separate plots from adjacent areas, preventing the lateral movement of water. Each of the 12 plots contained zones in which spruce trees neighboured other spruce trees (spruce zone), beech trees neighboured other beech trees (beech zone) and beech trees neighboured spruce trees in an interspecific contact zone (mixture zone) (Fig. 2). In 2013, six of the plots were assigned to the throughfall exclusion treatment group and equipped with retractable roofs, while the remaining six plots served as controls. The roofs were set to automatically close during rain events from 6 May, 2014 to 9 December, 2014, 10 March, 2015 to 21 November, 2015 and 6 March, 2016 to 4 November, 2016. The roofs remained open at all other times to minimise any changes in temperature and other stand conditions that were not related to precipitation, and remained open throughout winter. Air

157 temperature and precipitation levels were recorded at the Bavarian forest ecosystem monitoring
158 plot ca. 5 km west of Freising.

159 *Root, mycorrhiza and soil sampling*
160 Sampling was carried out once per year at the end of the vegetation period in the year before
161 throughfall exclusion (8 October, 2013), and before continuously opening the roofs during winter
162 in the years with throughfall exclusion (6 October, 2014, 12 October, 2015 and 2 November,
163 2015, and 4 October, 2016). Soil cores of 4-cm diameter were taken to a depth of 25 cm (2013,
164 2014) or 40 cm (2015, 2016) after removing any loose superficial litter. In each plot, one soil
165 core was obtained from each of beech and spruce zones, respectively , and two were obtained
166 from the mixture zone. Each soil core was separated into an upper part "topsoil" (average
167 thickness = 8.6 cm), which combined the $O_f + {}_hA_h$ horizons, and a lower part "deep layers" (>8.6
168 cm), which consisted of A_lB_v (KA5 classification; Eckelmann, Sponagel, & Grottenthaler, 2005).
169 Samples from the mixture zone were combined giving a total of six soil samples per plot. Each
170 sample was collected in a plastic bag, cooled immediately in the field and stored for up to 4
171 weeks at 4 °C until further processing. The root material within these samples was used to
172 examine ECM fungal community structure with two different approaches (morphotyping and
173 high throughput sequencing), and to measure exoenzyme activities.

174 *Soil parameters*
175 The volumetric soil water content was measured continuously using a time–domain
176 reflectometer (TDR 100; Campbell Scientific, Logan, Utah, USA). With vertical installation, the
177 probe signal integrated the soil water content over a soil depth of 10–30 cm. Therefore, the
178 uppermost probes were installed horizontally, integrating the signal over the top 0–7 cm of
179 mineral topsoil. One TDR probe was installed at both depths within each of the three zones

(beech, spruce and mixture; Fig. 2) in each of the 12 plots (n_{total} = 72). The sensor signals of all probes were assessed weekly throughout the year.

Fine-root parameters

Roots were manually separated from the soil, cleaned in tap water, and sorted under a stereomicroscope into beech and spruce roots. Samples were named according to tree species and zone, giving four sample types: spruce roots from spruce zones (SS), spruce roots from mixture zones (SMix), beech roots from beech zones (BB) and beech roots from mixture zones (BMix). Depending on the amount and vitality of fine-roots in a respective soil sample, either the entire sample (when few roots were present) or a subsample (in case many roots were present) was used for morphotype assessments and EA as detailed below. Subsampling was used to assure processing of one sample within one hour thus assuring comparability between different samples. Subsamples were generated by cutting all fine-roots (<1-mm diameter for beech; <2-mm diameter for spruce) of one sample into pieces of 2-cm length and by randomly picking a representative subsample (50 %, 33 % or 25 % of the total sample). 21 ECM tips per sample, respectively subsample were used up for enzyme activity assays and morphotype identification by Sanger sequencing. All remaining fine roots of each sample were stored below −20 °C and subsequently used for DNA extraction and high-throughput sequencing.

Ectomycorrhizal morphotype diversity and abundance

Vital mycorrhizal tips were assigned to morphotypes according to the colour and surface properties of the mycorrhizal mantle, and were categorised into exploration types according to Agerer (2001). The number of each morphotype was counted and used to calculate morphotype abundance per unit volume of soil. The ECM tips collected for enzyme activity measurements were frozen at −20 °C after finishing the assays for later identification according to their internal

transcribed spacer (ITS) ribosomal DNA (rDNA) sequence using polymerase chain reaction (PCR) and Sanger sequencing (see Method S1), resulting in several sequences per morphotype and year. These sequences were checked and assembled with CodonCode Aligner (CodonCode, Centerville, MA, USA) and contigs were submitted to BLAST searches against the UNITE database (Kõljalg et al., 2013) and the International Nucleotide Sequence Database (INSD). We only used the first entry of blast results and defined the following criteria to assign OTUs to species records: (1) sequence similarity ≥95 % and (2) a BLAST e-value $<2\times10^{-31}$. If more than 50 % the sequences of one morphotype yielded different species, but same genus, we used the genus information and if more than 50 % of the sequences of one morphotype yielded different genera, we kept our internal morphotype numbering (e.g. MT_18).

To distinguish between ECM fungal ability of potential water transport over several cm distance, we assigned the ECM tips to the following three exploration type groups that indicate soil exploration by extramatrical mycelia: (1) contact types (soil exploration radius 0 mm, no emanating hyphae), (2) short-distance (soil exploration radius up to 5 mm, some emanating hyphae) and medium-distance types (soil exploration radius up to 3 cm; fringe types: fans of emanating hyphae, mat types: undifferentiated rhizomorphs, smooth types: slightly differentiated rhizomorphs) and (3) long-distance types (soil exploration radius up to several dm, mostly highly differentiated rhizomorphs).

Potential extracellular enzyme activities of ectomycorrhizae

Twenty-one vital ECM tips were randomly selected from each sample, placed on wet filter paper and stored at 4 °C overnight. The number of tips of each morphotype was chosen according to its relative abundance in the sample, but was not lower than three. This design allowed the direct calculation of a weighted mean of EAs in each sample:

$$\overline{\mathrm{EA}}_{\text{per tip}} = \frac{\sum_n \mathrm{EA}}{n}, \quad (1)$$

where *n* is the number of ECM tips assayed per sample. This value was then further normalised to the number of ECM tips that occurred per unit volume of soil:

$$\mathrm{EA}_{\text{per vol}} = \frac{\overline{\mathrm{EA}}_{\text{per tip}} \times n}{\text{soil volume}_{\text{sample}}}, \quad (2)$$

where *n* is the number of vital ECM tips in a particular sample, representing the total EA in the sample.

The entire assay followed the procedure of Pritsch et al. (2011). In brief, seven substrates bound to 4-methylumbelliferone (MU) or aminomethylcoumarin (AMC) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were used to detect EAs: L-leucine-7-AMC (Leu-AMC) for the detection of leucine aminopeptidase (EC 3.4.11.1), 4-MU-β-d-xylopyranoside (MU-X) for xylosidase (EC 3.2.1.37), 4-MU-β-d d-glucuronide hydrate (MU-GU) for glucuronidase (EC 3.2.1.31), 4-MU-β-d-cellobioside (MU-C) for cellobiohydrolase (EC 3.2.1.91), 4-MU-N-acetyl-β-glucosaminide (MU-NAG) for N-acetyl-glucosaminidase (EC 3.2.1.14), 4-MU-β-d-glucopyranoside (MU-G) for β-glucosidase (EC 3.2.1.3), 4-MU-phosphate (MU-P) for phosphatase (EC 3.1.3.2) and ABTS for laccase (EC 1.10.3.2). Individual ECM tips were placed in the wells of 96-well filter plates (AcroPrep Advance 96, 30–40 µm PP/PE, 350 µL well, NTRL; Pall, Ann Arbor, MI, USA) and incubated with the respective substrates. Following filtration of the reaction solutions, the fluorescence (AMC and MU substrates) or absorption (ABTS) was measured. All assayed ECM tips were scanned to determine their projection area using the software WinRHIZO (Reg 2013e 32 Bit; Regent Instruments, Canada) and then immediately frozen for later identification according to their ITS rDNA.

Sample processing for high-throughput sequencing

The frozen fine roots from each sample were ground separately in liquid nitrogen, giving 384 samples [4 years × 2 soil depths × 12 (6 control, 6 throughfall exclusion) plots × 4 sample types (BB, SS, BMix, SMix)]. Contamination was controlled with extraction and PCR from negative controls. Approximately 350–450 mg of homogenate (or the total amount when less material was found) was used for DNA extraction with PowerSoil®-htp96 and PowerSoil DNA Isolation Kits (Mo-Bio, Carlsbad, CA, USA) following the manufacturer's instructions, with some modification for the initial bead beating as pre-experiments had shown very different levels of cell disruption, DNA yields and PCR success between samples. Frozen homogenates were transferred to 2-mL screw cap vials containing 5-mm steel beads, 600 µL PowerSoil bead solution and 60 µL PowerSoil C1-buffer from the kit, and were processed with a disruptor (2 × 30 s, 5000 rpm; Precellys24, Rockville, MD, USA) to separate the root tissues and hyphae into microparticles. The steel beads were then magnetically removed from the vials and replaced by garnets from the kit (1 g per sample), following which 150 µL PowerSoil bead-solution was added and shaken twice for 10 min at 20 Hz in a TissueLyser II (Qiagen, Hilden, Germany) to further disrupt the cells. The PowerSoil manufacturer's protocol was then followed. The resulting DNA was stored below −20 °C.

Amplification of ITS2 rDNA was performed with PCR primer mixes optimised for maximum phylogenetic recovery (Tedersoo et al. 2014, 2015; Table S2). All primers carried the respective forward or reverse overhang adapter sequences for the Illumina Miseq workflow (protocol Part # 15044223; Illumina, San Diego, CA, USA). Reactions consisted of 1 µL DNA (5 ng), 0.5 µL ITS3 mix (10 pmol equimolar mix of ITS3-Mix1 to -Mix5), 0.5 µL ITS4 mix (10 pmol equimolar mix of ITS4-Mix1 to ITS4-Mix4), 10 µL NEBNext® High-Fidelity 2X PCR Master Mix (New England Biolabs, Frankfurt, Germany) and 8 µL H_2O. PCR conditions were 5

269 min at 95 °C, 28 × [30 s at 95 °C, 30 s at 55 °C and 60 s at 72 °C] and 10 min at 72 °C. The
270 quality of all products was checked on agarose gels. Triplicate samples from successful PCRs
271 were pooled and cleaned using Agencourt AMPure XP (Beckman Coulter, Krefeld, Germany)
272 with a bead:DNA ratio of 1. Removal of primer dimers was controlled with the Bioanalyzer
273 DNA1000 Kit (Agilent Technologies, Waldbronn, Germany) and yield was quantified using the
274 Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen, Paisley, UK).
275 Indexing for multiplexed sequencing was performed using eight PCR cycles with individual
276 dual-index combinations of Nextera XT Index Kit v2 Sets A–D (Illumina). Reactions consisted
277 of 1 µL DNA (5 ng), 2.5 µL Primer 1 (Nextera i7 series), 2.5 µL Primer 2 (Nextera i5 series),
278 12.5 µL NEBNext High-Fidelity 2X PCR Master Mix and 6.5 µL H_2O. Indexed amplicons were
279 cleaned, size-checked and quantified as above. The amplicons (4 nM) from each sample were
280 pooled and rechecked with a Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies). The
281 final preparations and sequencing (Miseq v3 chemistry, 600 cycles flow cell, Illumina) followed
282 the manufacturer's recommendations for 16S Metagenomic Sequencing Library Preparation
283 (protocol Part # 15044223 Rev. B).

284 *Processing of high-throughput sequencing reads*
285 Data were obtained as demultiplexed FASTQ files and processed using the fungal ITS analysis
286 pipeline PIPITS v1.3.6 (Gweon et al., 2015) on Biolinux v8.0.6 (Field et al., 2006). Sequence
287 processing followed Gweon et al. (2015): read pairs were joined with PEAR v0.9.10
288 (parameters: -q 30; Q33, *P*-value of assembly ≤0.0001; Zhang et al., 2014) and
289 FASTQ_QUALITY_FILTER (parameters: -q 30, -p 80; Q33; FASTX-Toolkit,
290 http://hannonlab.cshl.edu, accessed 12 February, 2017); ITS2 of fungal origin was extracted with
291 ITSX v1.0.11 (Bengtsson-Palme et al., 2013); sequences <100 bp were removed and operational

292 taxonomic units (OTUs) were clustered by 97% sequence identity with VSEARCH v2.1.2
293 (https://github.com/torognes/vsearch/, accessed 12 February, 2017); chimera were removed
294 using the UNITE UCHIME reference dataset (v01.01.2016; http://unite.ut.ee/repository.php,
295 accessed 12 February, 2017); and reads were mapped onto OTUs, singletons were removed and
296 the taxonomy of OTUs was assigned with RDP Classifier v2.12 (Wang et al. 2007) against a
297 reference set of fungal ITS data (UNITE 7.1; Kõljalg et al. 2013). OTU and phylotype
298 abundance tables were then produced, whereby OTUs were defined as 'clusters of reads with
299 user-defined thresholds' and phylotypes were defined as 'clusters of sequences binned into the
300 same taxonomic assignments' (Gweon et al., 2015). Phylotypes were used for all further analyses
301 of high-throughput sequencing data, because phylotypes better resemble data obtained by
302 morphotyping of ECM fungi. Taxonomic assignments with a confidence threshold <0.85 were
303 omitted. Finally, all phylotypes were given a status that reflected whether they were ECM-
304 forming and their exploration type during a manual review guided by Agerer (2001) and
305 Tedersoo and Smith (2013).

306 *Statistical analyses*
307 All values are presented as means ± standard errors unless otherwise indicated.

308 For the morphotyping data, diversity indices were calculated using the package
309 BiodiversityR (Kindt, 2016) in R (R Core Team, 2016). The effects of throughfall exclusion, tree
310 species, competitive situation and soil depth on ECM fungal species abundances, diversity
311 indices and extracellular enzyme activities were analysed with analysis of variance (ANOVA)
312 using the software IBM SPSS Statistics 19 (IBM, Armonk, NY, USA). In this analysis, the effect
313 of the two tree species growing in three different species mixture situations was partitioned into
314 three orthogonal contrasts: (I) BMix and SMix vs. BB and SS; (II) BMix vs. SMix; (III) BB vs.

315 SS. As a measure of effect size, we calculated ω^2 (Hays, 1963). Detailed comparisons between
316 subsets of the data were conducted in R using unpaired two-sample *t*-tests where the data were
317 normally distributed (Shapiro test) or Wilcoxon signed-rank tests. To test the correlation between
318 the extracellular enzyme activities of the ECM tips and soil parameters and morphotype
319 abundances, Spearman's rank correlation coefficients were computed in R. The average
320 contribution of each species to the average overall Bray–Curtis dissimilarity was assessed by
321 calculating the similarity percentage (Clarke, 1993) in BiodiversityR. Differences in variation
322 between study years and treatment were tested with mixed effect models that considered plots as
323 a random factor, using the R package nlme (Pinheiro, Bates, DebRoy, & Sarkar, 2014).

324 Prior to further analysis of the high-throughput sequencing phylotype data, five samples with
325 low sequencing depth (<17,000 sequences per sample) were removed from the dataset, as well as
326 rare non-fungal or unassignable phylotypes as determined during the taxonomic assignment step.
327 The sequence reads were then randomly rarefied 10^4 times using GUniFrac for R (Chen, 2012)
328 and the results were averaged to compare all samples at equivalent sequencing depths (Weiss et
329 al., 2015). Bray–Curtis dissimilarities between the samples (ECM fungal community variation)
330 and Shannon diversity indices (ECM phylotypes only) were calculated using the vegan package
331 (Oksanen et al., 2017). Taxonomic overviews and ordinations were produced with the phyloseq
332 package (McMurdie & Holmes, 2013), and multivariate testing for the effect of environmental
333 characteristics on the ECM fungal community was conducted using Bray–Curtis dissimilarity
334 matrices with Adonis (permutational multivariate ANOVA using distance matrices; 10^5
335 permutations) in vegan. Statistical analyses that mirrored those for the morphotyping data were
336 performed as described above.

Results

Soil moisture

Throughfall exclusion decreased the volumetric soil water content during the vegetation period in the topsoil (0–7-cm depth), from ca. 30% in 2013 to ca. 10% in 2016. There was also a significant reduction of volumetric soil water content in the deep layers (10–30-cm depth) during the second and third throughfall exclusion period in 2015 and 2016, from ca. 35% in 2013 to 20–25% under beech and to 15–20% under spruce (Fig. 3).

ECM fungal community composition

In total, 45,181 vital ECM tips were counted and categorised into 43 morphotypes, from which 25 species were identified by their ITS rDNA. Three morphotypes did not yield evaluable sequences. On average, four ECM morphotypes were found per sample (minimum = 1, maximum = 11; see Table S3 for ECM morphotype abundances and distributions).

High-throughput sequencing yielded 18×10^6 quality-filtered reads, which were assigned to 4,820 OTUs and 1,411 phylotypes. Eleven samples were removed (five because of a low sequencing depth and six because of a low number of roots), leaving 373 samples for further analysis. The median abundance of fungal reads was 42,937 sequences per sample (minimum = 17,280, maximum = 103,220). In total, 144 phylotypes were identified as ECM fungi during manual inspection of all phylotypes following normalisation to an equal sequencing depth. On average, 11 ECM phylotypes were found per sample (Table S4).

In 2013 (i.e. 1 year before throughfall exclusion), there was no significant difference in the measures of ECM fungal community composition between the control and throughfall exclusion plots. On the basis of morphotypes, drought was a strong predictor for the abundance of the contact and short- and medium-distance exploration type groups. Repeated summer droughts led

to a progressive decline in contact types relative to control (decline by 67 ± 27% in 2014 ($P <$ 0.05), by 64 ± 27% in 2015 ($P < 0.01$), by 83 ± 21% in 2016 ($P < 0.01$), and a strong decline relative to control in short-distance and medium-distance types (decline by 54 ± 28% in 2014, by 83 ± 21% in 2015, by 96 ± 11% in 2016; $P < 0.05$ in all years) causing a strong increase in relative abundance of long-distance types relative to the other types (Fig. S5). By contrast, long-distance types were not affected in the first 2 years of drought, significantly decreasing in abundance relative to control only after 3 years (decline by 88 ± 18% in 2016, $P < 0.01$). Soil depth was also a major predictor for the abundance of all three exploration type groups, with ca. 90% vital tips occurring in the topsoil. In throughfall exclusion plots, changes in abundance and the proportion of exploration types were stronger and occurred earlier in the topsoil (throughfall exclusion × soil depth interaction: $P < 0.05$ in all years except for long-distance types in 2014 and 2015). By contrast, on the basis of phylotype data, drought was a weak predictor for the abundance of exploration type groups, with only the abundance of short-distance and medium-distance types being decreased significantly in the final year (decline by 72 ± 25% compared to control in 2016, $P < 0.01$).

The morphotype and phylotype Shannon diversity indices were quite similar (Fig. 4), but the phylotype diversity indices showed a smaller decline following repeated droughts. In the control plots, Shannon diversity indices were generally higher in the topsoil than in the deep layers (morphotype: 1.24 ± 0.04 vs. 0.92 ± 0.06, respectively, $P < 0.05$ in all years; phylotype: 1.39 ± 0.04 vs. 1.23 ± 0.05, respectively, $P < 0.05$ in 2014; Table 1). The effect of throughfall exclusion on ECM fungal diversity indices differed between beech and spruce, irrespective of the competitive situation. In the throughfall exclusion plots, the ECM fungal diversity indices remained unchanged in the first 2 years of drought for beech but declined from the second year

of drought (2015) onwards for spruce. In 2016, there was a significant difference in the morphotype diversity indices between beech (control: 1.01 ± 0.08; throughfall exclusion: 0.80 ± 0.02) and spruce (control: 0.93 ± 0.21; throughfall exclusion: 0.27 ± 0.11) depending on the treatment (throughfall exclusion × BB vs. SS interaction: $P < 0.01$), with a much stronger decline being observed in spruce. Phylotype diversity indices responded less to drought, but also exhibited a pronounced decline in the SS samples after the third drought period in 2016. After 3 years of drought, throughfall exclusion had a smaller effect on morphotype diversity indices of ECM fungal communities from the mixture zone (0.82 ± 0.09 to 0.57 ± 0.14) than on those from the spruce and beech zones (1.11 ± 0.09 to 0.53 ± 0.11; throughfall exclusion × BMix and SMix vs. SS and BB interaction: $P < 0.05$).

The composition of ECM fungal communities (phylotypes and morphotypes) also changed gradually following throughfall exclusion, with differences becoming apparent after three successive drought years (phylotypes: Fig. 5). While there was no significant difference between the ECM fungal communities in the control and throughfall exclusion plots in 2013 before the start of the experiment, they became distinct after 3 consecutive years of throughfall exclusion [Adonis $R^2_{adjusted}$ (phylotypes) = 0.03 (2013), 0.02 (2014), 0.01 (2015) and 0.16** (2016); Adonis $R^2_{adjusted}$ (morphotypes) = -0.02 (2013), 0.09* (2014), 0.08 (2015) and 0.21** (2016) with * $P < 0.05$, ** $P < 0.01$]. The effects of a species mixture on drought tolerance differed among the four most frequent ECM fungal species that were shared by both tree species. For example, the absolute morphotype abundances of *Cenococcum geophilum* and *Russula ochroleuca* were not affected by drought and not influenced by the competitive situation or tree species, whereas the morphotype abundances of *Lactarius tabidus* and *Xerocomus pruinatus* were negatively

affected by drought but this was less pronounced when the tree species grew in mixed situation compared to beech from beech samples (Fig. S6).

Potential extracellular enzyme activities

Overall, the most pronounced effect of throughfall exclusion on EAs and differences between the qualitative measure $EA_{per\ tip}$ and the quantitative measure $EA_{per\ vol}$ was observed in the topsoil. As visualised by the regression lines of EA from throughfall exclusion vs. control plots, $EA_{per\ tip}$ (Fig. 6a) was remarkably stable in spruce and beech ectomycorrhizae (except for laccase as detailed below) while $EA_{per\ vol}$ (Fig. 6b) decreased over repeated drought years, which was mainly caused by a decline of vital ectomycorrhizae on throughfall exclusion plots (Fig. 6c).

In detail, there was no significant difference in $EA_{per\ tip}$ of the seven tested hydrolytic enzymes between throughfall exclusion and control plots in 2013 (prior to the treatment) and in the first 2 years of throughfall exclusion (2014 and 2015). Even after three drought periods, in 2016 $EA_{per\ tip}$ of only three out of seven hydrolytic enzymes changed significantly: xylosidase had significantly higher levels in the throughfall exclusion plots than in the control plots (4.43 ± 0.74 vs. 2.89 ± 0.48 pmol cm^{-2} min^{-1}, respectively), and the cellulose-degrading enzymes cellobiohydrolase and β-glucosidase exhibited a greater increase following drought in the mixture zone than in the beech and spruce zones (Table S7). By contrast, the $EA_{per\ tip}$ of laccase (which releases nutrients bound to phenolic compounds) was significantly lower in throughfall exclusion plots than in control plots from 2014 onwards, and was also decreased in control plots compared to the other years in the naturally dry year 2015 (2014: 90.53 ± 15.09 vs. 199.17 ± 33.19; 2015: 26.17 ± 4.36 vs. 27.75 ± 4.62; 2016: 94.76 ± 15.80 vs. 151.12 ± 25.19 mmol cm^{-2} min^{-1}, respectively). Laccase activity disappeared in spruce ECM from the topsoil of the spruce zones in 2016 along with laccase-positive morphotypes. Comparison of the influence of the

contrasting study years (2014–2016) and the throughfall exclusion treatment on $EA_{per\ tip}$ showed variations between years to be greater than between treatments, except for leucine aminopeptidase. The activity of this enzyme was increased (yet not significantly: $P = 0.077$) by treatment over the years.

ANOVA (Table S8) revealed that there were few interactions between throughfall exclusion and other factors (zone, soil depth) for the $EA_{per\ tip}$ data from 2015 onwards. There was, however, a significant interaction between throughfall exclusion and soil depth for xylosidase, glucuronidase and chitinase in 2015 and for phosphatase in 2016, reflecting an increase in EA in the deep layers of drought plots but no change in the topsoil (Table S7). Furthermore, significant interactions between throughfall exclusion, soil depth and mixture situation were found for cellobiohydrolase in 2015, and for cellobiohydrolase, β-glucosidase and laccase in 2016 (Table S8), with the $EA_{per\ tip}$ of cellobiohydrolase and β-glucosidase being higher in control plots than in throughfall exclusion plots in the topsoil of the mixture zone. In 2016, the $EA_{per\ tip}$ for laccase in the topsoil increased in the beech and spruce zones but declined in the mixture zone, while that in the deep layers decreased in the beech and spruce zones and exhibited no significant change in the mixture zone in throughfall exclusion plots (Table S7). The interaction between throughfall exclusion and soil depth and species mixtures became significant only in the third year of throughfall exclusion, with leucine aminopeptidase and phosphatase being decreased in SMix samples and not affected (phosphatase) or stimulated (leucine aminopeptidase) in BMix samples in the topsoil, and exhibiting the opposite response in the deep layers in 2016 (Table S8).

In contrast to qualitative stability of $EA_{per\ tip}$, $EA_{per\ vol}$ showed a progressive decline on throughfall exclusion plots over repeated drought years. Before the onset of treatment (2013), there were no significant differences in $EA_{per\ vol}$ between control and throughfall exclusion plots,

but after three consecutive summer droughts, the $EA_{per\ vol}$ of both tree species significantly declined in the throughfall exclusion plots (Table S7). Xylosidase, cellobiohydrolase, β-glucosidase, glucuronidase and phosphatase were significantly altered from 2015 onwards, while N-acetyl-glucosaminidase and leucine aminopeptidase significantly changed in 2016 (Table S8).

In the deep layers, $EA_{per\ tip}$ of was also very stable but tended to increase in spruce ECM fungi with progressing drought (Fig. S9a). Soil depth significantly affected almost all $EA_{per\ vol}$ (Fig. S9b) in all years (Table S8), because on average there were 5–10 times fewer roots in the deeper layers than in the topsoil (Fig. 10c). $EA_{per\ vol}$ of beech ECM continuously decreased during repeated drought whereas $EA_{per\ vol}$ of spruce ECM did not respond with a clear increase or decrease (Fig. S9b). The relative decline in vital ECM tips in throughfall exclusion plots compared to control plots was less pronounced in the deep layers than in the topsoil. Vital ECM tips of spruce only declined in 2016 (Fig. S9c) as reflected in the respective diversity indices (Fig. 4).

There were significant interactions between soil depth and throughfall exclusion in 2014 and 2015, indicating the effects of faster drying in shallower soil (Fig. 3). Most interactions with soil depth were transient and (with the exception of phosphatase) disappeared in 2016 when the deeper soil had dried more thoroughly (Table S8). The zones within a plot tended to have a larger effect on enzyme activities at the beginning of the experiment, with this effect disappearing with repeated throughfall exclusion (Table S8). Only phosphatase and laccase $EA_{per\ vol}$ showed a zone effect in 2016. Overall, $EA_{per\ vol}$ declined more strongly in SMix samples compared to SS samples (Table S7).

The EAs of some ECM fungi became dominant under throughfall exclusion, i.e. *L. tabidus* in both tree species, *Russula fellea* in beech and *C. geophilum* in spruce, mainly because other morphotypes disappeared. These species were also identified as making a high (>10%, SIMPER $P < 0.05$) contribution to the differences in ECM morphotype community between control and throughfall exclusion plots.

Discussion

Do repeated years of throughfall exclusion influence ECM fungal community composition and functions more strongly in spruce than in beech?

While shifts in ECM fungal community composition after drought have repeatedly been reported (Cavender-Bares et al., 2009; Shi et al., 2002), a reduced ECM fungal diversity as in our study has rarely been detected (Swaty et al., 2004). Shannon diversity of ECM fungal communities from the beech and spruce zones reflected the contrasting strategies of beech and spruce to cope with drought. Beech exhibited a decline in ECM fungal diversity after the first year of drought and then maintained a slightly lower level than the control, which supports the previous finding that beech continues to produce new fine roots during drought (Burkhardt & Pariyar, 2016; Nikolova et al., 2010) allowing the surviving ECM fungi to colonize newly growing roots. By contrast, ECM fungal diversity did not exhibit a marked change in spruce following the first drought, but declined dramatically after the second drought year. After one severe summer drought Nikolova et al. (2010) found that spruce sustained standing fine roots rather than growing new ones, supporting a strategy of decreased growth during drought conditions (Dobson et al., 1990; Maier-Maercker, 1998). Our results of a decline in ECM fungal Shannon diversity in spruce suggest that on the longer term, this strategy would prevent new colonisation by ECM fungi and, over several years, lead to a decline in diversity. This indicates that spruce is

495 particularly vulnerable to predicted future climate change scenarios for those areas in the
496 observed climate zone that are prone to repeated summer drought (Zang et al., 2014), supporting
497 our first hypothesis.

498 The changes in Shannon diversity did not directly translate to losses in qualitative enzymatic
499 potentials of ECM fungal communities which further supports presence of highly
500 complementary and functionally redundant hydrolytic enzyme activities in ECM fungal
501 communities even under severe drought (Buée, Courty, Mignot, & Garbaye, 2007; Courty,
502 Pritsch, Schloter, Hartmann, & Garbaye, 2005; Jones et al., 2010). However, both $EA_{per\ tip}$ and
503 $EA_{per\ vol}$ of laccase were strongly decreased in ECM fungal communities in throughfall exclusion
504 plots already from the first year of throughfall exclusion onwards and in control plots in the
505 naturally dry year 2015, suggesting that ECM fungi expressing laccase activity were drought
506 sensitive at the Kranzberg site. Activity of the oxidative enzyme laccase is very widespread in
507 the fungal kingdom (Iyer & Chattoo, 2003; Junghanns, Moeder, Krauss Martin, & Schlosser,
508 2005; Vasconcelos et al., 2000) with several functions in degradation but also morphogenesis
509 (e.g. Baldrian, 2006, Thurston, 1994). Oxidase activities in soil are more dynamic than
510 hydrolytic activities (Sinsabaugh et al., 2008), corresponding to our observations. In ECM fungi
511 laccase is related to the release of nutrients (particularly N) enclosed in recalcitrant polymers or
512 protein-phenol complexes (Baldrian, 2006). Whether the strong decline in ectomycorrhizae with
513 laccase enzyme activity causes lasting effects on nutrient relations in forest soil will depend on
514 how long it takes for the full functional spectrum in ECM fungal communities to be restored
515 following drought release, and on how other soil fungal groups are affected by drought. In ECM
516 fungi, laccase is only present in some lineages (Luis et al., 2005) and by selecting dominant
517 morphotypes in EA measurements, we likely excluded ECM fungal taxa that became relatively

518 rare as a consequence of the decline in vital fine roots. The decline in vital fine roots leading to a
519 complete loss of formerly dominant ECM fungi with laccase activity again supports our first
520 hypothesis of stronger drought effects on spruce ECM fungal community composition and
521 functions.

522 *Does repeated drought lead to changes in the functionality of the ECM fine-root system towards*
523 *traits that are related to drought resistance, irrespective of the tree species?*
524 We observed a decrease in contact type ectomycorrhizae following drought, confirming the
525 findings of Bakker et al. (2006), who interpreted this as caused by shrinking soils and thus
526 reduced contact with the substrate. In addition, the dominant contact types at our plots were
527 *Lactarius* spp. with thin cell walls prone to loosing cellular integrity under dry conditions (di
528 Pietro et al., 2007) which makes them sensitive to drought. The relative increase in long-distance
529 type mycorrhizae in both tree species following drought suggests that they have higher drought
530 resistance because of their ability to explore and transport water beyond the root surface
531 (Cairney, 1992; Duddridge et al., 1980). The direction of changes in exploration types as
532 functional traits i.e. increasing long-distance type and decreasing contact and short-distance type
533 ectomycorrhizae was the same in both tree species, thus supporting our second hypothesis.

534 Relative stability of extracellular enzyme activities has often been observed in ECM fungal
535 communities upon environmental disturbance (Diedhiou et al., 2010; Jones et al. 2010), which
536 underlines the importance of finding alterations in three enzymes in the present experiment in the
537 third year of throughfall exclusion. One enzyme activity (leucine aminopeptidase) was strongly
538 increased in ECM fungal communities of both tree species under drought (albeit in different
539 mixture situations). Interestingly, a stimulation of this EA under a strong drought was also
540 observed in *C. geophilum* ectomycorrhizae associated with different *Quercus* species (Herzog et

al. 2013). In that study, $EAs_{per\ tip}$ of the other enzymes measured (the same six enzymes as in our study) showed neutral responses. Herzog et al. (2013) found that the decrease in abundance of *C. geophilum* was negatively correlated with leucine aminopeptidase activity and suggested that this EA had to be compensated for by an increased activity of the remaining vital tips of *C. geophilum*. Our results suggest a similar mechanism at the whole ECM fungal community level.

Drought increased the extracellular cellulolytic potential per vital tip irrespective of tree species. Extracellular cellulolytic activity may be stimulated by the presence of dead fine root material, which accumulated during repeated drought events, to gain access to nutrients contained in these dead tissues (Hupperts, Karst, Pritsch, & Landhäusser, 2017; Lindahl & Tunlid, 2015; Pritsch & Garbaye, 2011). The alternative explanation of saprotrophic carbon acquisition by ECM fungi from organic matter decay rather than the internal carbon supply of the plant (Bréda et al., 2013; Courty, Bréda, & Garbaye, 2007) seems unlikely as the observed increase in long-distance types under drought suggests that carbon was not limiting. These findings indicate an overall qualitative preservation of functionality in ECM fungal communities at the level of vital root tips. However, a decline in the number of vital tips led to quantitative functional losses in ECM fungal communities at the ecosystem level.

Thus, structural diversity supported our second hypothesis that repeated drought leads to changes in the functionality of the ECM fine-root system towards traits that are related to drought resistance, irrespective of the tree species at the vital root tip and ecosystem level, while enzyme activities did not support it at the ecosystem level.

561 *Does tree mixture attenuate negative effects of drought on ECM fungal communities of beech*
562 *and spruce compared with monospecific stands?*
563 Growth in mixed stands had significant positive effects on morphotype diversity indices of
564 the ECM fungal communities of both tree species after 3 years of throughfall exclusion, at which
565 time the low soil water content indicated rather high stress levels (Davidson, Belk, & Boone,
566 1998). This supports the stress gradient hypothesis of increased facilitation among species with
567 increasing stress levels (Bertness & Callaway, 1994). To determine whether these emerging
568 facilitation effects would continue, further sampling is required with increasing stress levels.
569 Thus, our third hypothesis was only preliminarily supported by the morphotype data. A probable
570 reason for the observed higher morphotype diversity in the mixture zones could be reduced
571 competition for non-limiting resources due to different soil exploration of the two tree species
572 (Bolte & Villanueva, 2006).

573 ECM fungi colonising both tree species may contribute to resource partitioning between
574 fungi and different trees, facilitating stress resistance (Beiler, Durall, Simard, Maxwell, &
575 Kretzer, 2010). Among those ECM fungi were a contact and a long-distance type species. The
576 decline of these two abundant species upon drought was reduced in the mixture zones. This
577 suggests that mixture provides vital ectomycorrhizae with different functional attributes as
578 starting material for recolonising newly grown roots during recovery after drought: in our study
579 this was indicated by drought-tolerance among the four most frequent morphotypes shared by
580 beech and spruce. This indicates mixture to increase resilience of forest ecosystems after
581 drought.

582 The hydrolytic $EA_{per\ tip}$ was maintained even after two consecutive summers with prolonged
583 drought periods, with mixture effects only becoming apparent in the third year of throughfall

584 exclusion. This can probably be attributed to changes in niche occupation by roots of the two tree
585 species, as indicated by the reduction in some enzyme activities (leucine aminopeptidase and
586 phosphatase) in spruce from mixture but not in beech from mixture in the topsoil, and their
587 increase in the deep layers. $EA_{per\ vol}$ did not exhibit any mixture effects. Thus, according to the
588 assumption that 3 years of throughfall exclusion evoked strong stress on ECM fungal
589 communities, we do not accept H3 according to enzyme activity data.

590 *Overall implications of repeated drought on below-ground functioning of forest ecosystems*
591 Regarding nutrient cycling in forest soils under repeated drought, our results suggest that the
592 potential to forage for nutrients contained in organic materials is retained in surviving
593 ectomycorrhizae. Moreover, preferential carbon allocation of trees to ECM fine roots upon
594 recovery from drought has recently been demonstrated to be an important mechanism for
595 restoring fine root functionality in forest ecosystems (Hagedorn et al., 2016).

596 In soils, low moisture leads to low EAs *in situ* due to impaired diffusion processes and
597 death/inactivity of decomposers, which in turn lead to a retardation of decay processes and
598 thereby to an accumulation of substrate (van der Molen et al., 2011; Brando et al., 2008). Upon
599 rewetting, high amounts of substrate meet a functional ECM fungal community and stimulate the
600 recovery of soil microbial processes (Hagedorn et al., 2016). An increasing amount of dead
601 ectomycorrhizal fine roots may lead to a retardation of decay processes in forest soils and is
602 currently debated to either increase or decrease carbon stocks in forest ecosystems (see
603 Fernandez & Kennedy, 2016 for a review). However, low water availability is likely more
604 growth limiting in these temperate systems than nutrient limitation (Sardans & Peñuelas 2005)
605 because spring and autumn still provide time and water for mineralising organic compounds in
606 temperate regions struck by summer drought. Thus, also phases of recovery from drought may be

607 critical to assess when nutrient relations in forest ecosystems are considered after severe drought
608 (Geßler, Schaub, & McDowell, 2017; Hagedorn et al., 2016).

609 *Methodological implications*

610 The different numbers and abundances of morphotypes and phylotypes in our study resulted
611 from known methodological constraints of high-throughput sequencing, which overestimates
612 diversity by including the DNA of non-vital ECM fungi, single hyphae and resting stages
613 (Medinger et al., 2010). By contrast, morphotyping is prone to underestimating species richness
614 even when including ITS rDNA information as it cannot distinguish between visually similar
615 ectomycorrhizae (Erland, Jonsson, Mahmood, & Finlay, 1999). However, manual morphotyping
616 allows direct observations of degree of mycorrhization and vitality of ectomycorrhizae and fine
617 roots. Shannon diversity was remarkably similar for morphotypes and phylotypes, indicating that
618 both methods provide similar basic ecological information on ECM fungal community
619 composition. However, because vital and non-vital ECM tips were not distinguished, high-
620 throughput sequencing results reveal a potential rather than actual community composition.
621 Therefore, RNA-based approaches (Baldrian et al., 2012; van der Linde & Haller, 2013) should
622 be used to assess the active ECM fungal community via high-throughput sequencing.

623 *Vulnerability of temperate forests under drought*

624 In this study, we experimentally applied drought stress to a habitat that was not adapted to
625 repeated summer droughts. The combined analysis of ECM fungal community diversity and
626 functional traits suggested that correlations between enzyme activities and ECM fungal species
627 varied depending on the interplay between throughfall exclusion, tree species interaction and soil
628 depth. Such context dependency has also been reported in several previous studies on ECM
629 fungal communities, as reviewed in Bahram, Peay, and Tedersoo (2015). However, by subjecting

this mesic forest ecosystem to repeated summer droughts, we were able to detect a strong reduction in enzymatic activities and ECM fungal abundances at the ecosystem level because of fine root die-back under water shortage. We showed that niche complementarity may be important in attenuating the effects of repeated summer droughts on ECM fungal communities in beech-spruce mixtures. One important mechanism of niche complementarity may be the redistribution of water to shallow soil layers by hydraulic lift.

Our findings underline the vulnerability of temperate forests and similar Holarctic ecosystems to prolonged and frequent summer droughts (Allen et al., 2010). Therefore, we advocate long-term experiments when studying forest ecosystems in the context of drought and support the assertion that mesic forests are endangered by long-term drought (Young et al., 2017). Such experiments would allow us to explore whether ECM fungal communities develop further mechanisms for drought tolerance depending on their habitat, how the same ECM fungal species from dry and moist sites perform under repeated droughts and how they influence host tree performance. This may guide future forest management in areas with predicted alterations in precipitation regimes.

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Conflict of interest

The authors declare no conflicts of interest.

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944

Tables

Table 1 Effect size ω² (explained variance) of an analysis of variance (ANOVA) examining the effect of throughfall exclusion (TE), zone [three orthogonal contrasts: roots of beech and spruce from monospecific and mixed stands], soil layer (topsoil vs. deep layers) and their interactions on the Shannon diversity index (H′). H′ (morphotypes): calculated from morphotype data; H′ (phylotypes): calculated from high-throughput sequencing phylotypes. Values of ω² with $P < 0.05$ are written in bold. Adjusted R^2 values for the respective ANOVA models are given in the last row of the table.

Source of variation	H′ (morphotypes)				H′ (phylotypes)			
	2013	2014	2015	2016	2013	2014	2015	2016
throughfall exclusion (TE)	0.080	**0.277**	**0.637**	**0.475**	0.010	-0.047	0.068	0.134
zone	-0.004	0.084	0.077	**0.263**	**0.205**	0.072	-0.059	**0.236**
BMix and SMix vs. BB and SS	0.018	-0.023	0.084	0.080	0.034	0.071	-0.021	-0.011
BMix vs. SMix	-0.001	0.007	-0.031	**0.121**	**0.101**	0.040	-0.023	-0.013
BB vs. SS	-0.019	**0.096**	0.029	**0.125**	**0.126**	-0.027	-0.019	**0.254**
TE x zone	-0.078	-0.022	-0.002	**0.206**	-0.050	**0.164**	-0.060	-0.035
TE × BMix and SMix vs. BB and SS	-0.029	-0.031	-0.028	**0.119**	-0.025	**0.141**	-0.021	-0.025
TE × BMix vs. SMix	-0.030	-0.020	0.004	-0.007	-0.022	-0.006	-0.026	-0.022
TE × BB vs. SS	-0.023	0.023	0.030	**0.171**	-0.014	0.048	-0.020	0.001
soil layer	**0.163**	**0.153**	**0.132**	**0.121**	0.037	**0.096**	-0.016	-0.013
soil layer × TE	-0.007	**0.055**	**0.085**	-0.017	-0.014	**0.058**	0.028	-0.012
soil layer × zone	-0.024	0.075	-0.039	0.111	-0.056	-0.001	0.008	0.073
soil layer × BMix and SMix vs. BB and SS	-0.017	0.050	-0.009	-0.008	-0.015	-0.014	0.027	-0.001
soil layer × BMix vs. SMix	0.001	0.033	-0.020	0.123	-0.022	0.032	-0.009	**0.095**
soil layer × BB vs. SS	-0.008	-0.012	-0.009	-0.011	-0.020	-0.014	-0.017	-0.013
soil layer× TE × zone	-0.004	-0.006	0.055	-0.023	-0.050	0.057	0.001	-0.031
soil layer × TE × BMix and SMix vs. BB and SS	0.009	0.011	0.027	0.000	-0.006	0.030	0.033	-0.006
soil layer × TE × BMix vs. SMix	-0.015	-0.005	0.009	-0.003	-0.023	-0.013	-0.023	-0.014
soil layer × TE × BB vs. SS	0.000	-0.002	0.020	-0.011	-0.022	0.041	-0.006	-0.022
$R^2_{adjusted}$	0.346	0.417	0.396	0.368	0.309	0.201	–0.126	0.312

954 **Figure captions**

955 **Fig. 1** Mean temperature and precipitation at the experimental site in Kranzberg Forest from 1
956 year before the start of the experiment. The *y*-axes are scaled after Walter and Lieth (1960) so
957 that precipitation < temperature indicates an arid month (e.g. July 2013 and 2015) and
958 precipitation > temperature indicates a humid month. Precipitation: light, growing season;
959 medium, dormant season; dark, five-fold compression of the precipitation axis. Arrows indicate
960 sampling dates (8 October, 2013, 6 October, 2014, 12 October, 2015 and 2 November, 2015, and
961 4 October, 2016).

962 **Fig. 2** Schematic diagram of the sampling zones in the plots. Spruce, zone of spruce
963 neighbouring spruce; mixture, interspecific contact zone between beech and spruce; beech, zone
964 of beech neighbouring beech; modified from Goisser et al. (2016).

965 **Fig. 3** Volumetric soil water content at the experimental site in Kranzberg Forest measured at
966 depths of **(a)** 0–7 cm and **(b)** 10–30 cm with time–domain reflectometer (TDR) probes.
967 Throughfall exclusion periods were from 6 May, 2014 to 9 December, 2014, 10 March, 2015 to
968 21 November, 2015 and 6 March, 2016 to 4 November, 2016 (see Fig. 1). Values were averaged
969 for throughfall exclusion plots (red) and control plots (dark blue) for mixture zones (solid lines),
970 beech zones (dashed lines) and spruce zones (dotted lines). Shaded areas indicate the standard
971 deviations; marks along the *x*-axis indicate the measurement dates.

972 **Fig. 4** Ectomycorrhizal (ECM) fungal diversity (Shannon diversity H′) in the topsoil **(a)** and the
973 deeper layers **(b)** before (2013) and during 3 consecutive years of throughfall exclusion during
974 the vegetation period. Plots were subdivided into zones in which beech and spruce were
975 surrounded by the same species (BB, SS) or neighboured the other species (BMix, SMix). Error

bars: ± 1 se; dark blue lines and circles: control plots (n = 6); red lines and triangles: throughfall exclusion plots (n = 6); H′ (MT): calculated from data according to morphotypes; H′ (PT): calculated from high-throughput sequencing phylotypes; sampling dates: 8 October, 2013, 6 October, 2014, 12 October, 2015, 2 November, 2015 and 4 October, 2016.

Fig. 5 Non-metric multidimensional scaling (NMDS) plots showing changes in the ectomycorrhizal (ECM) phylotypes before (2013) and during 3 consecutive years with (red) or without (blue) throughfall exclusion during the vegetation periods (2014–2016). Dots represent single root samples; distances represent differences in ECM fungal community composition based on Bray–Curtis dissimilarities. Density lines were plotted according to the distribution of the samples in the graph using the function geom_density2d() from the package ggplot2 (Wickham, 2009) in R.

Fig. 6 Potential enzyme activities (EAs): **(a)** $EA_{per\ tip}$ (pmol cm^{-2} min^{-1}) as weighted mean of EA per ectomycorrhizal (ECM) tip in an ECM community (see materials and methods equation 1), **(b)** $EA_{per\ vol}$ (pmol cm^{-2} min^{-1} cm^{-3}) taking into account the number of vital ECM tips per soil volume (see materials and methods equation 2), and **(c)** number of vital ectomycorrhizae of spruce and beech in topsoil samples over four study years (2013-2016). EA values of the respective same sample type in control and throughfall exclusion plots (Table S7) were log transformed and plotted against each other for each year separately to illustrate overall effects of throughfall exclusion. Linear regressions were calculated and plotted for these pairs per year with the colour code from darker in 2013 to lighter in 2016. The grey dashed line with a slope of 1 and an intercept of 0 was drawn to indicate when EAs under control are equal to EAs under throughfall exclusion. Deviation of the slope of regression lines from 1 with an intercept remaining close to 0 indicate similar relative degrees and directions of change in all EAs,

whereas shift in the intercept indicates that EA values changed to different degrees and/or directions. Values of intercept and slope are given in the top left corner of each panel with asterisks indicating significant differences from a slope of 1 and an intercept of 0 (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). For ease of visualization, the different enzymes were not specifically indicated in this representation, and EA values were plotted without standard error (for detailed values see Table S7). Symbols represent sample types (circles BB, squares BMix, triangles SS, diamonds SMix) resulting in four values per enzyme and a total of 28 values per year of seven hydrolytic enzymes (xylosidase, cellobiohydrolase, β-glucosidase, chitinase, leucine aminopeptidase, phosphatase and glucuronidase). From the eight studied EAs, laccase was excluded as it showed a clearly different behaviour compared to the seven hydrolytic enzymes (Fig. S10). Error bars in panel (c) indicate ± 1 standard error.

Supporting Information captions

Method S1 Internal transcribed spacer (ITS) determination for ectomycorrhizal (ECM) morphotypes.

Table S2 Primer sequences for high-throughput sequencing.

Table S3 Abundance of morphotypes.

Table S4 Abundance of high-throughput sequencing phylotypes.

Figure S5 Relative abundance of each exploration type group.

Figure S6 Changes in shared ectomycorrhizal (ECM) species for beech and spruce between years.

Table S7 Measured values of the mean enzymatic activities in each sample ($EA_{per\ tip}$) and the mean enzymatic activity normalised to the number of tips per unit volume of soil ($EA_{per\ vol}$).

Table S8 Analysis of variance (ANOVA) table for the mean enzymatic activity in each sample ($EA_{per\ tip}$) and the mean enzymatic activity in each sample normalised to the number of tips per unit volume of soil ($EA_{per\ vol}$).

Figure S9 Mean potential enzyme activities ($EA_{per\ tip}$) and mean enzyme activity normalised to the number of tips per unit volume of soil ($EA_{per\ vol}$) of the ectomycorrhizal fungal communities and loss of vital ECM tips in the deep layers.

Figure S10 Principal component analysis (PCA) of mean enzymatic activity ($EA_{per\ tip}$) and mean enzymatic activity in normalised to the number of tips per unit volume of soil ($EA_{per\ vol}$).

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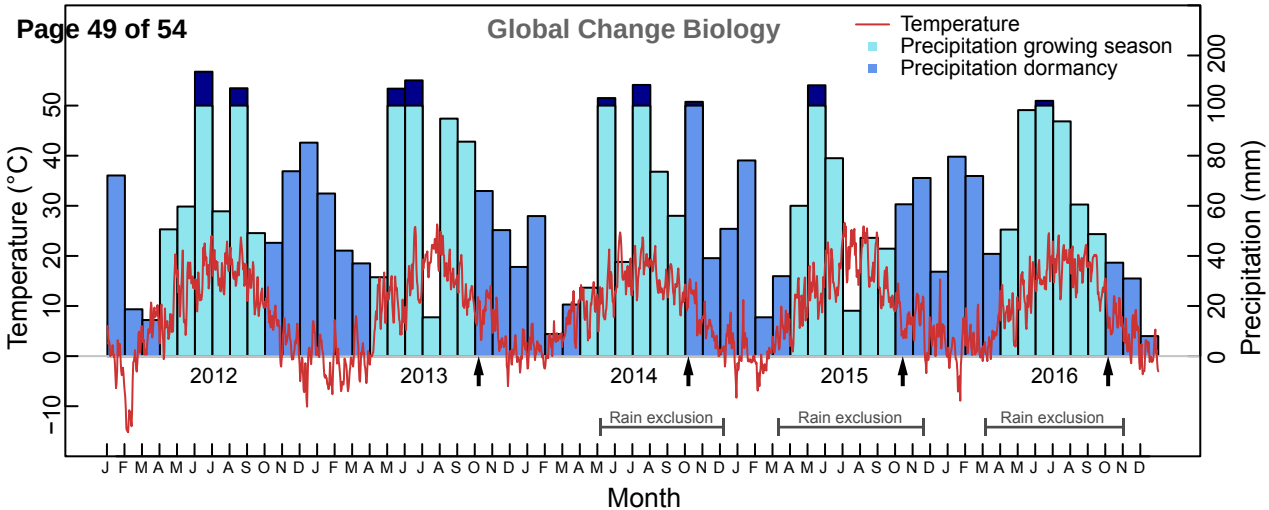


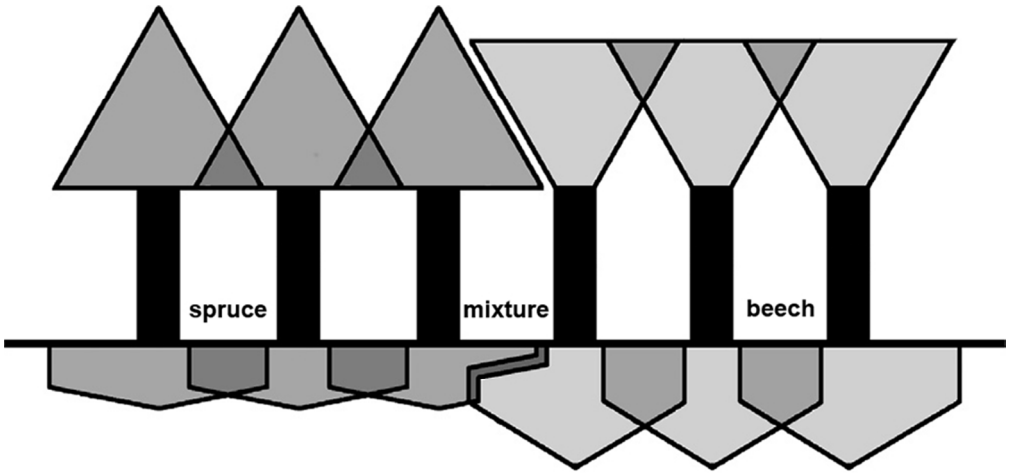
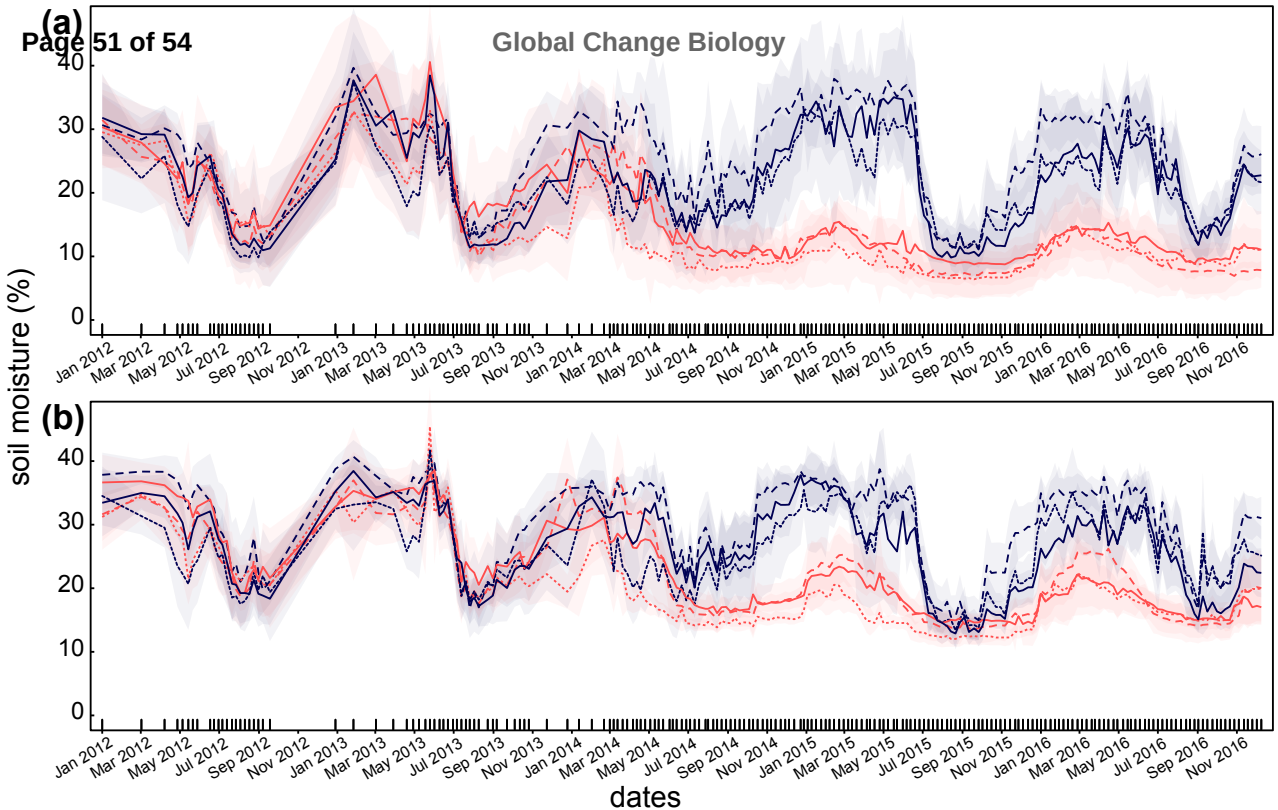
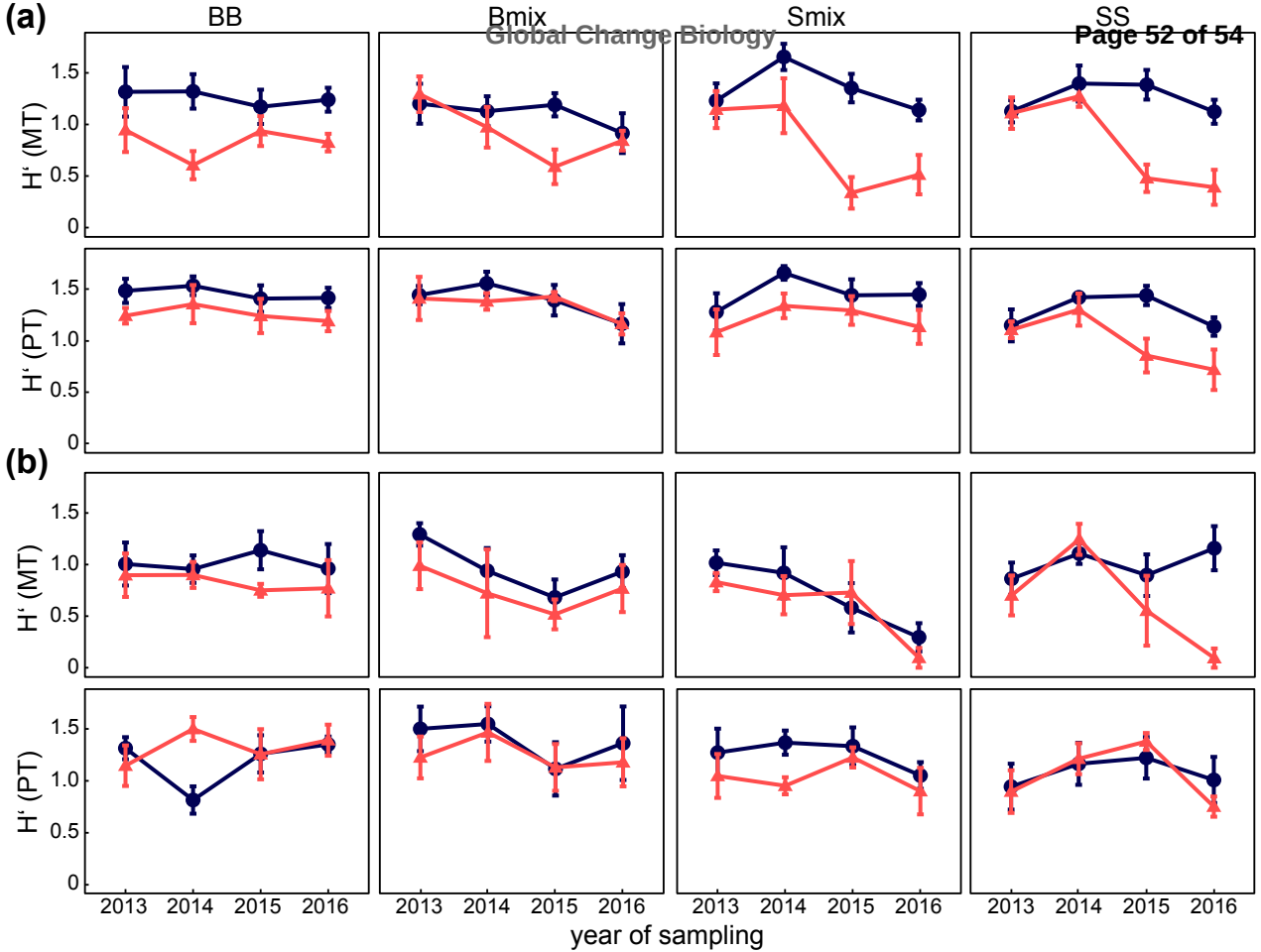
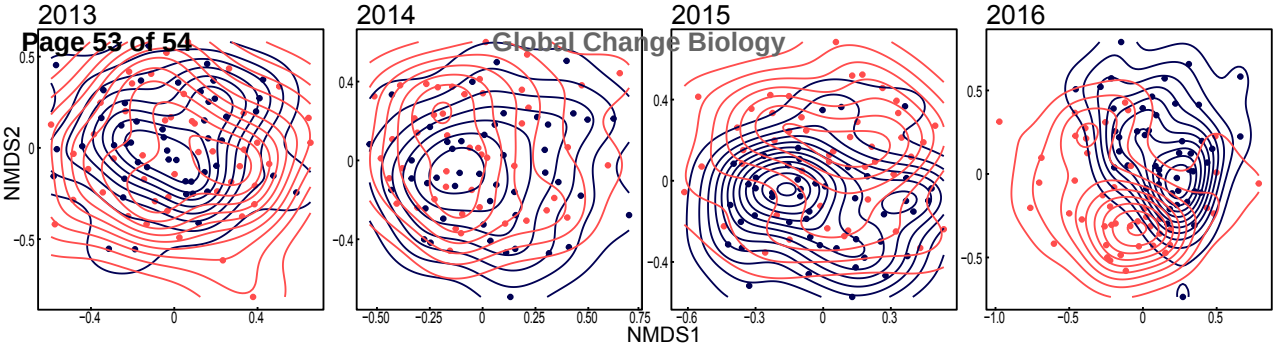


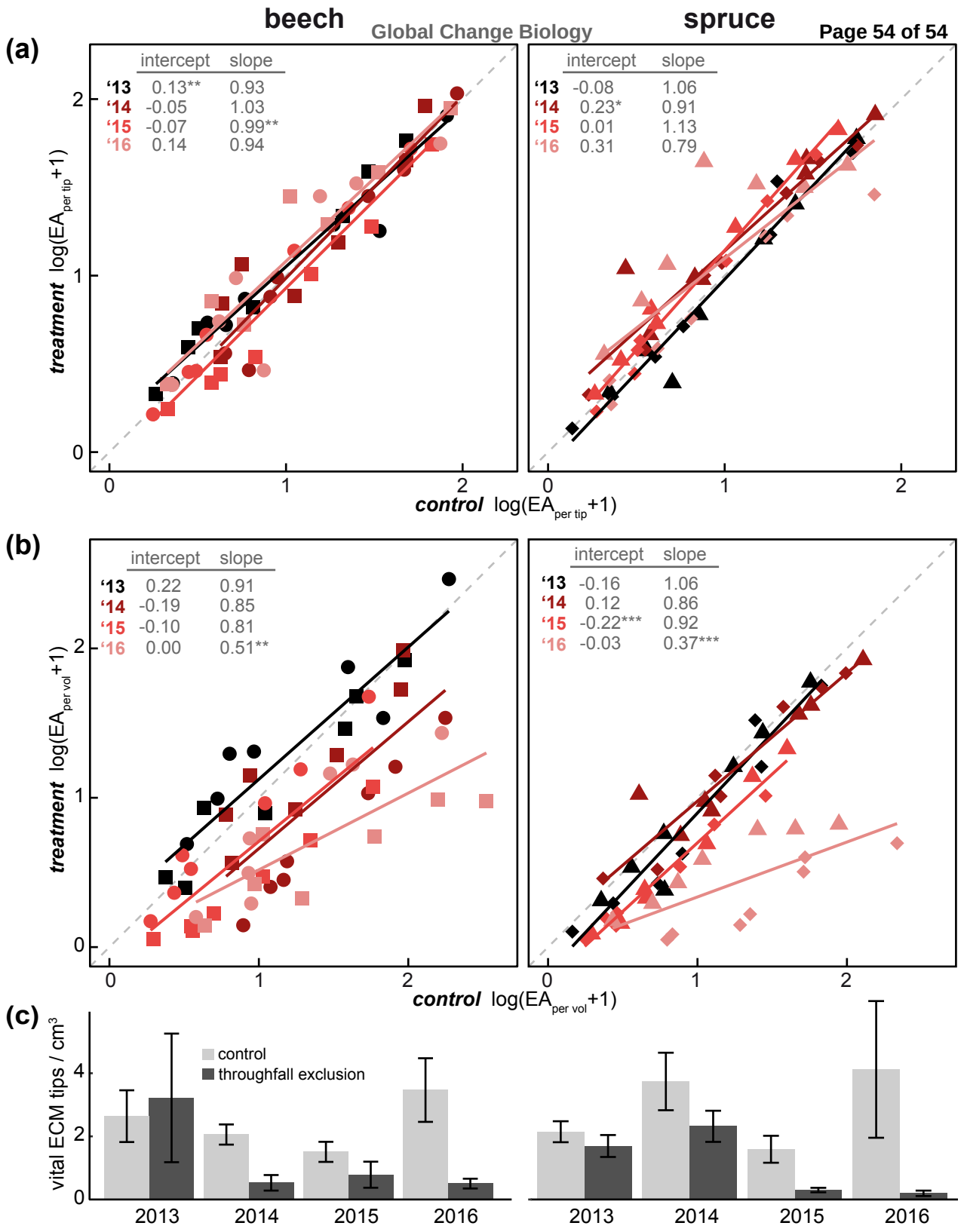
Fig. 2 Schematic diagram of the sampling zones in the plots. Spruce, zone of spruce neighbouring spruce; mixture, interspecific contact zone between beech and spruce; beech, zone of beech neighbouring beech; modified from Goisser et al. (2016).

80x37mm (300 x 300 DPI)


(a)
(b)
soil moisture (%)
0
10
20
30
40
Jan 2012
Mar 2012
May 2012
Jul 2012
Sep 2012
Nov 2012
Jan 2013
Mar 2013
May 2013
Jul 2013
Sep 2013
Nov 2013
Jan 2014
Mar 2014
May 2014
Jul 2014
Sep 2014
Nov 2014
Jan 2015
Mar 2015
May 2015
Jul 2015
Sep 2015
Nov 2015
Jan 2016
Mar 2016
May 2016
Jul 2016
Sep 2016
Nov 2016
dates


(a)
(b)
BB
Bmix
Smix
SS
H' (MT)
H' (PT)
0
0.5
1.0
1.5
2013
2014
2015
2016
year of sampling


2013
2014
2015
2016
NMDS2
NMDS1
0.5
0
−0.5
0.4
−0.4
−0.50
−0.25
0.25
0.50
0.75
−0.6
−0.3
0.3
−1.0
1.0


(a)
beech
spruce
intercept slope
'13 0.13** 0.93
'14 -0.05 1.03
'15 -0.07 0.99**
'16 0.14 0.94
intercept slope
'13 -0.08 1.06
'14 0.23* 0.91
'15 0.01 1.13
'16 0.31 0.79
treatment log(EA per tip +1)
control log(EA per tip +1)
(b)
intercept slope
'13 0.22 0.91
'14 -0.19 0.85
'15 -0.10 0.81
'16 0.00 0.51**
intercept slope
'13 -0.16 1.06
'14 0.12 0.86
'15 -0.22*** 0.92
'16 -0.03 0.37***
treatment log(EA per vol +1)
control log(EA per vol +1)
(c)
vital ECM tips / cm3
control
throughfall exclusion
2013
2014
2015
2016