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Elevated temperature may reduce functional but not taxonomic diversity of fungal assemblages on decomposing leaf litter in streams

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Abstract

Mounting evidence points to a linkage between biodiversity and ecosystem functioning (B-EF). Global drivers, such as warming and nutrient enrichment, can alter species richness and composition of aquatic fungal assemblages associated with leaf-litter decomposition, a key ecosystem process in headwater streams. However, effects of biodiversity changes on ecosystem functions might be countered by the presumed high functional redundancy of fungal species. Here, we examined how environmental variables and leaf-litter traits (based on leaf chemistry) affect taxonomic and functional α- and β-diversity of fungal decomposers. We analysed taxonomic diversity (DNA-fingerprinting profiles) and functional diversity (community-level physiological profiles) of fungal communities in four leaf-litter species from four subregions differing in stream-water characteristics and riparian vegetation. We hypothesized that increasing stream-water temperature and nutrients would alter taxonomic diversity more than functional diversity due to the functional redundancy among aquatic fungi. Contrary to our expectations, fungal taxonomic diversity varied little with stream-water characteristics across subregions, and instead taxon replacement occurred. Overall taxonomic β-diversity was fourfold higher than functional diversity, suggesting a high degree of functional redundancy among aquatic fungi. Elevated temperature appeared to boost assemblage uniqueness by increasing β-diversity while the increase in nutrient concentrations appeared to homogenize fungal assemblages. Functional richness showed a negative relationship with temperature. Nonetheless, a positive relationship between leaf-litter decomposition and functional richness suggests higher carbon use efficiency of fungal communities in cold waters.

KEYWORDS
α-diversity, β-diversity, aquatic hyphomycetes, community physiological profiles, DNA fingerprinting, functional redundancy, leaf-litter decomposition

1 | INTRODUCTION

Global change impacts on biogeochemical cycles and habitats (Steffen et al., 2015) are triggering shifts in community composition through changes in species distribution and/or extinctions (Pereira et al., 2010). Species loss is predicted to increase continuously, exerting adverse effects on ecological processes, given the dependence of ecosystem functioning on biodiversity (biodiversity–ecosystem function; B-EF) (Cardinale et al., 2006; Hooper et al., 2012; Loreau et al., 2001). Mounting evidence supports positive B-EF relationships across ecosystems (Boyero, López-Rojo, et al., 2021; Boyero, Pérez, et al., 2021; Maestre et al., 2012; Mori et al., 2016; Raviraja et al., 2006), although this has not always been found (e.g. Dang et al., 2005; López-Rojo et al., 2019; Rubio-Ríos et al., 2021). The enhancement of ecosystem functions with species richness greatly depends on the functional differences among the species within assemblages as well as on the environmental context (Fetzer et al., 2015). Accordingly, at a local scale, functional diversity might be a better predictor of ecosystem functioning than taxonomic diversity (Reiss et al., 2009; Woodward, 2009).

Headwater streams are particularly vulnerable to global-change-related stressors (e.g. climate change, species invasions and pollution) that are threatening biodiversity and the integrity of ecosystems (Reid et al., 2019; Woodward et al., 2010). Streams in forested areas greatly depend on terrestrial plant detritus, primarily leaf litter (Webster et al., 1999); in these ecosystems, aquatic hyphomycetes are key intermediaries of energy and nutrients to higher trophic levels (Gessner et al., 2010; Graça et al., 2015). Therefore, alterations in community composition and/or species loss of aquatic hyphomycetes may have consequences on leaf-litter decomposition and nutrient cycling in stream food webs. Fungal assemblage composition and/or diversity in streams can be affected by temperature (Bärlocher et al., 2008; Fernandes et al., 2012; Ferreira & Chauvet, 2011a) and nutrient concentrations in water (Duarte et al., 2009; Jabiol et al., 2018). Fungal species richness tends to increase with moderate nutrient enrichment (Dunk et al., 2015). However, there is a controversy on the effects of temperature on taxonomic diversity of fungi involved in leaf-litter decomposition in streams (Bärlocher et al., 2008; Dang et al., 2009; Duarte et al., 2016; Fernandes et al., 2012), probably because fungal species differ in their cardinal temperatures.

Several B-EF studies point to a positive relationship between fungal diversity and leaf-litter decomposition (Bärlocher & Corkum, 2003; Pascoal et al., 2010; Raviraja et al., 2006), but others failed to detect such effects, pointing to a considerable functional redundancy among aquatic fungi (Andrade et al., 2016; Dang et al., 2005; Geraldes et al., 2012). Despite the recognized importance of biodiversity to stream ecosystem functioning (Gessner et al., 2010; Giller et al., 2004), most studies on leaf-litter decomposition addressed this issue by manipulating fungal assemblages (species richness and composition) in laboratory experiments (e.g. Geraldes et al., 2012; Gonçalves et al., 2015) or by comparing taxonomic diversity in reference *vs* impacted sites at reduced spatial scales (e.g. Duarte et al., 2008; Pascoal et al., 2005).

Physiological profiles have been used in terrestrial environments (e.g. Pinzari et al., 2016; Rinnan et al., 2009) to study fungal functionality (Dobranic & Zak, 1999). Carbon-source utilization profiles have been assessed from an array of carbon substrates as a mean of assessing fungal assemblage functionality, representing a standardized measure that allows functional comparisons across a variety of ecosystems (Sobek & Zak, 2003). This technique has been used in headwater streams to address bacterial functionality (Gionchetta et al., 2020; Ylla et al., 2014). However, to the best of our knowledge, it has not been applied to test the metabolic functionality of fungal decomposers of leaf litter in streams, and it might be useful to further clarify B-EF relationships.

In this study, we examined B-EF relationships focusing on taxonomic and functional diversity of fungal decomposers in streams with different environmental contexts. We hypothesize that stream-water temperature and dissolved nutrients will alter (i) taxonomic diversity (α-diversity) and/or (ii) composition of fungal assemblages (β-diversity). Moreover, we hypothesize that (iii) functional diversity will be maintained across different environmental contexts if functional redundancy among fungal species occurs and (iv) decomposition will be positively related to functional rather than to taxonomic diversity. To test these hypotheses, we carried out an *in situ* leaf-litter decomposition experiment in streams of four Mediterranean subregions differing in water temperature and chemistry, and riparian plant species composition. Four leaf-litter species with contrasting traits, one species representative of each subregion, were incubated in all streams across the subregions. Taxonomic and functional diversities were measured to unravel the potential drivers of changes in B-EF relationships of fungal decomposers.

2 | MATERIALS AND METHODS

2.1 | Study area

We selected low-order (first to second order) streams from four subregions in Andalusia (Southern Spain): Sierra Nevada, Sierra de Cazorla, Alcornocales and the Semiarid Lowland of Almería. Four permanent streams were selected in each subregion (total 16 streams, see Table S1). These areas are under different nature protection status and show marked biogeoclimatic contrast. Riparian vegetation is dominated by deciduous tree species in the coldest subregions (Sierra Nevada and Sierra de Cazorla), by a mixture of deciduous trees and broad-leaf evergreen shrubs in Alcornocales, and by giant graminoids in the Semiarid Lowland of Almería (Salinas et al., 2018).

2.2 | Environmental characterization of streams

Electrical conductivity (EC), pH and dissolved oxygen were measured in each stream with a multiparametric probe (HACH® model HQ-30d, Loveland, CO, USA). Total alkalinity was measured by acid titration to a pH endpoint of 4.25 (Wetzel & Likens, 2000). Total

dissolved nitrogen (TN) and phosphorus (TP), nitrates (NO_3–N) and soluble reactive phosphorus (SRP) were measured. An aliquot of 100 ml of non-filtered water was wet mineralized for 30 min at 120°C in an autoclave. After cooling to room temperature, an aliquot of 50 ml was acidified with concentrated sulphuric acid to determine TN (absorbance at 220 nm), whereas TP, mineralized to phosphate, was determined in the remaining 50 ml (Wetzel & Likens, 2000). Dissolved inorganic nutrients were analysed from the filtered samples (0.45 μm, APFC, Merk Millipore): NO_3-N, by the sodium salicylate method (APHA, 2005), and soluble reactive phosphorus by the ascorbic acid method (Wetzel & Likens, 2000). All measurements were performed twice in each stream, at the beginning and the end of the decomposition experiment. Water temperature was recorded hourly in each stream with HOBO Pendant® loggers (Onset Computer Corporation) during the full period of leaf-litter immersion in the streams.

2.3 | Leaf-litter chemistry and field experiment

We selected one native and/or dominant riparian leaf species from each of the four subregions to perform a leaf-litter decomposition experiment by reciprocal incubations across subregions. The four leaf species differed widely in litter traits: two were deciduous species, (i) the nitrogen-fixer alder (*Alnus glutinosa* [L.] Gaertn.) from Sierra Nevada and (ii) the narrow-leaved ash (*Fraxinus angustifolia* Vahl) from Sierra de Cazorla. The other two leaf species were (i) the broadleaf evergreen shrub rhododendron (*Rhododendron poncticum* sub sp. *baeticum* [Boiss. & Reut.] Hand.-Mazz.) from Alcornocales and (ii) the graminoid giant cane (*Arundo donax* L.) from Semiarid Lowland of Almería. Abscised leaves of each plant species were collected in autumn 2016 at the corresponding subregion. Toughness, specific leaf area (SLA) and percentages of hemicellulose, cellulose and lignin were determined as in Fenoy et al. (2016). Silica (Si) concentration was measured using ICP mass spectrometry (iCAP 6500—ICP-OES, Thermo Scientific®). Litter nitrogen (N) and carbon (C) concentrations were determined using a Perkin Elmer series II CHNS/O elemental analyzer (EA-Thermo DELTA V Advantage, Fisher Scientific®), with results expressed as % N and % C of litter dry mass. Phosphorus (% P) was determined following the method described in Wetzel and Likens (2000), after sample incineration (500°C, 5 h). Litter characterization was performed on leached material (3 litter bags per species that were incubated for 24 h in one stream per subregion).

Portions (5.0 ± 0.05 g dry mass) of each leaf-litter species were pre-moistened and introduced into fine-mesh bags (15 × 20 cm; 1 mm mesh size). Leaf bags (5 bags per species per stream) were deployed and tied to iron stakes anchored to the streambed in riffles along a 50 m stream reach. The experiment was carried out during winter and lasted for 40 days. After that, leaf bags were retrieved from streams, placed individually in zip-lock bags and transported to the laboratory in an icebox. In the laboratory, leaves were removed from the bags and carefully rinsed with filtered stream-water to eliminate fine particles. Then the leaves were oven-dried (70°C, 72 h) and weighed to the nearest 0.1 mg. Thereafter, the dried leaves were ground to pass a 1 mm screen; a portion was ignited at 500°C for 5 h to estimate ash-free dry mass (weighed to the nearest 0.1 mg). Leaf-litter decomposition was expressed as ash-free dry mass loss per degree-day (LML dd^{-1}).

An additional set of 5 litter bags per plant species per stream, incubated and processed as above, was used to measure taxonomic and functional diversity. After retrieval from the streams and the elimination of fine particles, a portion of leaf litter from each bag was frozen (−80°C) until the analyses of taxonomic diversity were performed, and the other portion was immediately used for the analyses of functional diversity (see below).

2.4 | Fungal taxonomic diversity

Taxonomic diversity of fungal assemblages was assessed as the number of operational taxonomic units (OTUs) from denaturing gradient gel electrophoresis (DGGE) of fungal DNA according to Duarte et al. (2010). Five leaf discs (1 cm Ø) from each of the 5 bags per species per stream were pooled (25 discs per sample) and used for genomic DNA extraction with DNeasy® PowerSoil® Kit (Quiagen), following the manufacturer's protocol. Fungal diversity was assessed using the primer pair ITS3GC and ITS4, which amplifies the ITS2 region of fungal rDNA. The forward primer contained 40 bp GC tail at the 5′ end to ensure the amplicon separation by DGGE. For polymerase chain reaction (PCR), 2 μl of extracted DNA was mixed with 1 μl (0.4 μM final conc.) of each primer, 1 μl (0.2 mM final conc.) of dNTP-mix, 6 μl (3 mM final conc.) of $MgCl_2$, 0.03 U (final conc.) of GoTaq® G2 Flexi DNA polymerase, 10 μl (1× final conc.) of Green GoTaq® Flexi buffer and 28.7 μl of nuclease-free ultrapure water. PCR was carried out in iCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) starting with initial denaturation at 95°C for 2 min, followed by 36 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s and elongation at 72°C for 1 min; and finishing with final elongation at 72°C for 5 min. DGGE was carried out in a DCode™ Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA) to separate the PCR products of similar length (~400 bp) differing in nucleotide compositions. Amplified fungal DNA (20 μl per sample) were loaded on 8% (w/v) polyacrylamide gel in 1× Tris-acetate-EDTA with a denaturing gradient from 30% to 70% (100% denaturant corresponds to 40% formamide and 7 M urea) and the DGGE was conducted at 55 V and 56°C for 16 h and stained with Midori Green (Grisp) for 10 min on a shaker at 40 rpm. Gel images were captured under UV in ChemiDoc™ XRS system (BioRad Laboratories).

2.5 | Community-level physiological profiles (CLPP)

Fungal functional diversity was evaluated using the Soil Fungi Log procedure, adapted from Sobek and Zak (2003). Portions of leaf

litter from mesh bags ($n = 5$) of the same species incubated in each stream were pooled and ground (T25 digital Ultra-Turrax®) in filtered (0.45 μm) water from the corresponding stream on an ice bath, and sieved through 500 μm and 250 μm mesh. To assess actual microbial community function, the inoculum was standardized by leaf-litter biomass (Garland, 1997; Preston-Mafham et al., 2002) because inoculum density is meaningless when single cells are not involved (Garland & Lehman, 1999). The wet weight of leaf-litter particles that resulted in the initial optical density (OD_{490}) lower than 0.25 (Sobek & Zak, 2003) was estimated previously (not shown). Leaf-litter particles were added to a sterilized solution of agar (15 ml, 0.2% w/v), made with filtered stream-water containing 200 μL of antibiotics from a stock solution (150 mg of streptomycin sulphate and 75 mg of chlortetracycline hydrochloride in 15 ml of sterile distilled water) to prevent bacterial activity. The particles and the antibiotic solution were transferred to a screw-capped glass tube containing the sterile agar solution, after allowing it to cool and gently agitated to obtain a homogenized suspension immediately before the inoculation to microtiter plates. A volume of 100 μl of this mixture was transferred to each well of the Biolog FF microtiter plates (Biolog, Hayward, California, USA) inside a laminar flow cabinet. These plates comprise a blank well and 95 wells with different carbon compounds plus tetrazolium violet, a redox dye to measure colorimetrically the mitochondrial activity resulting from substrate oxidation. C-sources included mainly carbohydrates and carboxylic acids, and also amino acids, amines/amides, polymers, and miscellaneous compounds. A complete list of carbon sources in FF microplates can be found elsewhere (see Preston-Mafham et al., 2002).

The FF microtiter plates were incubated at the mean winter temperature of each subregion. The OD_{490} of each well for each plate was measured immediately after inoculation (colourless) and in every 24 h until the value reached 2 in the well with higher colour development. The increase in OD_{490} of each individual well was calculated by subtracting its own initial value from the values measured along the incubation period, obtaining the net OD_{490} changes for each well. The average well colour development (AWCD) was calculated using the equation: $AWCD = [\Sigma (F - I)] / n$; that is, the sum of differences between final (F) and initial absorbance (I) over time divided by the number of substrates ($n = 95$) (Rinnan et al., 2009). To level off the effects of different incubation temperatures among samples of different subregions on colour development rates, data at a predefined AWCD of 0.15 were selected (Rinnan et al., 2009) for the analysis of substrate utilization patterns. With those data selected for each sample, we created a matrix of samples × intensity of individual C-source usage, from which functional richness, Shannon diversity and β-diversity were calculated.

2.6 | Data analyses

The gel images of DGGE were aligned and normalized; the bands in the gel were considered as operational taxonomic units (OTUs) and the relative intensities of the bands were analyzed with BioNumerics v5.0 software (Applied Maths, SintMartens-Latem, Belgium).

Fungal richness and Shannon diversity were calculated for taxonomic (OTUs) and functional (CLPP) data and assessed for differences among subregions and leaf-litter species using two-way ANOVA with *Anova* function from 'car' package. Pairwise comparisons of level means for a given factor within each level of the other factor were performed using the *multicomp* and *lsmeans* packages in R. We performed Spearman's rank correlation between taxonomic and functional richness to test for functional redundancy.

Regressions of taxonomic and functional local richness (per each stream) of fungi associated with leaf-litter species were carried out against the independent environmental variables and leaf-litter traits using general linear models (GLM) with a Poisson error distribution and a log link function. A subset of environmental variables mostly related to the global change drivers in stream water (temperature, NO_3^--N and soluble reactive phosphorus) were used as predictors. Electrical conductivity was also included in the analyses because of its high inter- and intra-subregional variability. Leaf-litter traits were standardized and summarized using the first two rotated components (RC1 and RC2 henceforth) of a Principal Component Analysis (Figure S1). Moreover, the same type of model was applied to investigate the spatial structure of response variables: taxonomic and functional local richness, using Moran's eigenvector map (MEM) variables as spatial predictors. MEM variables were generated using Principal Coordinates of Neighbour Matrices (PCNMs): a truncated Euclidean distance matrix was calculated from the geographical coordinates of the sampling sites to extract eigenvectors associated with positive eigenvalues, which can be used as explanatory variables in multiple regression analyses (Borcard & Legendre, 2002). The PCNM approach allows assessing spatial structures over the entire range of scales encompassed by the geographical sampling area. The first PCNMs represent broader spatial scales, and the last ones cover finer spatial structures (Borcard & Legendre, 2002).

To test for differences among subregions and litter species in taxonomic and functional composition, we carried out a permutational MANOVA based on dissimilarity matrices (with Hellinger distance) using the *adonis* function (with $n = 999$ permutations) in the 'vegan' package. The same dissimilarity matrices were used to calculate the overall taxonomic and functional β-diversities (β_{Total}): the average dissimilarity between pairs of samples (4 litter species incubated in 16 streams). To discriminate factors affecting fungal taxonomic or functional β_{Total} diversities, first the local contribution to both β_{Total} diversities (LCBD) was determined following Legendre and Cáceres (2013). Then, we evaluated how the set of leaf traits (RC1 and RC2 components of the rotated PCA) and the environmental variables related to global change stressors, and spatial variables (the same initial set of MEM variables used for richness, see above) affected taxonomic and functional LCBDs, using beta regression models (*betareg* function from 'betareg' package) with a logit link function. Because of the nature of LCBD data (i.e. values ranging from 0 to 1), we used beta regression to model our response variables (Cribari-Neto & Zeileis,

2010). β_{Total} and LCBD were computed with function *beta.div* from 'adespatial' package. Separate beta regression models were built for taxonomic and functional LCBD using as predictors litter-traits and environmental variables. Additionally, we also built a beta regression for both dependent variables, using spatial predictors (MEM variables) as independent factors to unravel whether site uniqueness could be spatially structured following the environmental gradients. Finally, the relationships of leaf mass loss (LML) per degree day (dd^{-1}) with functional richness and Shannon diversity were tested using linear models, after checking model assumptions (see above). These regressions were performed for the overall dataset (n = 64) after log-transformation of dependent variables.

All statistical analyses were performed in R (R Core Team, 2018).

3 | RESULTS

3.1 | Stream-water characteristics

Streams from different subregions greatly differed in their water characteristics, particularly temperature, electrical conductivity, alkalinity and nutrient concentrations (Table S1). The mean winter temperature of water was lower in Sierra Nevada (4.0 ± 0.3°C), intermediate in Sierra de Cazorla (7.7 ± 1.0°C) and Alcornocales (11.8 ± 0.4°C), and higher in Semiarid Lowland (16.9 ± 2.0°C). The streams from all subregions showed high level of dissolved oxygen (Table S1). Water chemistry was strongly related to lithology; the calcareous subregions (Sierra de Cazorla and Semiarid Lowland: >200 mg L^{-1} of $CaCO_3$) had higher pH (>8.0), and particularly higher electrical conductivity (5- to 32-fold) and total alkalinity (~8- to 14-fold) than the siliceous subregions (Alcornocales and Sierra Nevada). There were also 2- to 5-fold and 2- to 6-fold higher concentrations of total-N and NO_3^--N, respectively, in the calcareous subregions than the siliceous ones. Conversely, most of the streams in the siliceous subregions showed 1.5- to 2-fold higher phosphorus concentrations (Table S1).

3.2 | Leaf-litter traits

Leaf-litter species differed significantly in N content: % N was higher in *Alnus* (2.61 ± 0.05%), followed by *Fraxinus* (1.01 ± 0.00%), *Rhododendron* (0.65 ± 0.03%) and *Arundo* (0.41 ± 0.01%) (Table S2). *Arundo* had the highest content in cellulose (1.8-fold) and silicon (>50-fold), which could explain its higher toughness, but had lower C, P and lignin contents (1.3-, 1.6- and 4.6-fold, respectively) than the other leaf-litter species (Table S2). The first two rotated components (RC) of PCA on litter traits explained 91% of the total variance (Figure S1). Litter N (0.96), P (0.75) and lignin (0.75) showed a strong positive loading on RC1, while toughness (−0.88) and cellulose (−0.83) were negatively correlated with this dimension. RC2 was positively correlated with hemicellulose (0.97) and silicon (0.80) (Figure S1).

3.3 | Fungal taxonomic and functional richness across subregions and leaf-litter species

Fungal taxonomic richness and Shannon diversity (based on fungal DNA fingerprints, Table S3) did not differ among subregions or leaf-litter species, and the interactions between subregions and litter traits were not significant ($p > .05$; Table S4; Figure 1A and 1B). On the contrary, fungal functional richness differed significantly among subregions ($F_{3,48} = 5.43$, $p = .003$), being on average 8% higher (post-hoc, $p < .001$) in the colder subregions (Sierra Nevada and Sierra de Cazorla) compared to the warmer subregions (Alcornocales and Semiarid Lowland) (Figure 1C, Tables S3 and S5). Effects of leaf-litter species on fungal functional richness and Shannon diversity were significant but less pronounced than subregion effects (Figure 1C, Table S5). Fungal functional richness differed among litter species ($F_{3,48} = 5.08$, $p = .004$) and there was a significant interaction between litter species and subregion ($F_{9,48} = 2.53$, $p = .018$). Similarly, functional Shannon diversity differed among subregions ($F_{3,48} = 10.05$, $p < .001$), being on average 9% and 18% higher (post-hoc, $p < 0.001$) in Sierra Nevada and Sierra de Cazorla, respectively (colder subregions) than in the warmer subregions (Figure 1D, Table S5). Functional Shannon diversity varied within litter species depending on the subregion (Figure 1D, Table S5), that is, there was a significant interaction between litter species and subregion ($F_{9,48} = 2.79$, $p = .010$).

Taxonomic and functional richness were not correlated (rho = −0.09, *p*-value =.456), indicating that there was no relationship between the number of OTUs and the functional richness of the fungal assemblage.

3.4 | Fungal taxonomic and functional β-diversity across subregions and litter species

Fungal taxonomic composition (OTUs based on fungal DNA fingerprints) significantly differed among subregions (*adonis*, $F = 4.39$, $R^2 = 0.19$, $p < .001$), but no significant effects of litter species (*adonis*, $F = 0.97$, $R^2 = 0.04$, $p = .51$) or interaction (*adonis*, $F = 0.59$, $R^2 = 0.08$, $p = 1.0$) were found. Fungal functionality (based on community-level physiological profiles – CLPP) significantly differed among subregions (*adonis*, $F = 8.24$, $R^2 = 0.27$, $p < .001$) and litter species (*adonis*, $F = 1.63$, $R^2 = 0.05$, $p = .029$), and interactions between the subregions and litter species were found (*adonis*, $F = 1.56$, $R^2 = 0.15$, $p = .006$). Moreover, taxonomic β_{Total} (0.81) was fourfold higher than functional β_{Total} (0.20).

3.5 | Predictors of fungal richness and local contribution to β-diversity

Fungal functional richness was negatively affected by temperature and PCNM1, which represented variation at the largest spatial scale across subregions (Table 1). The regression model for local contribution to

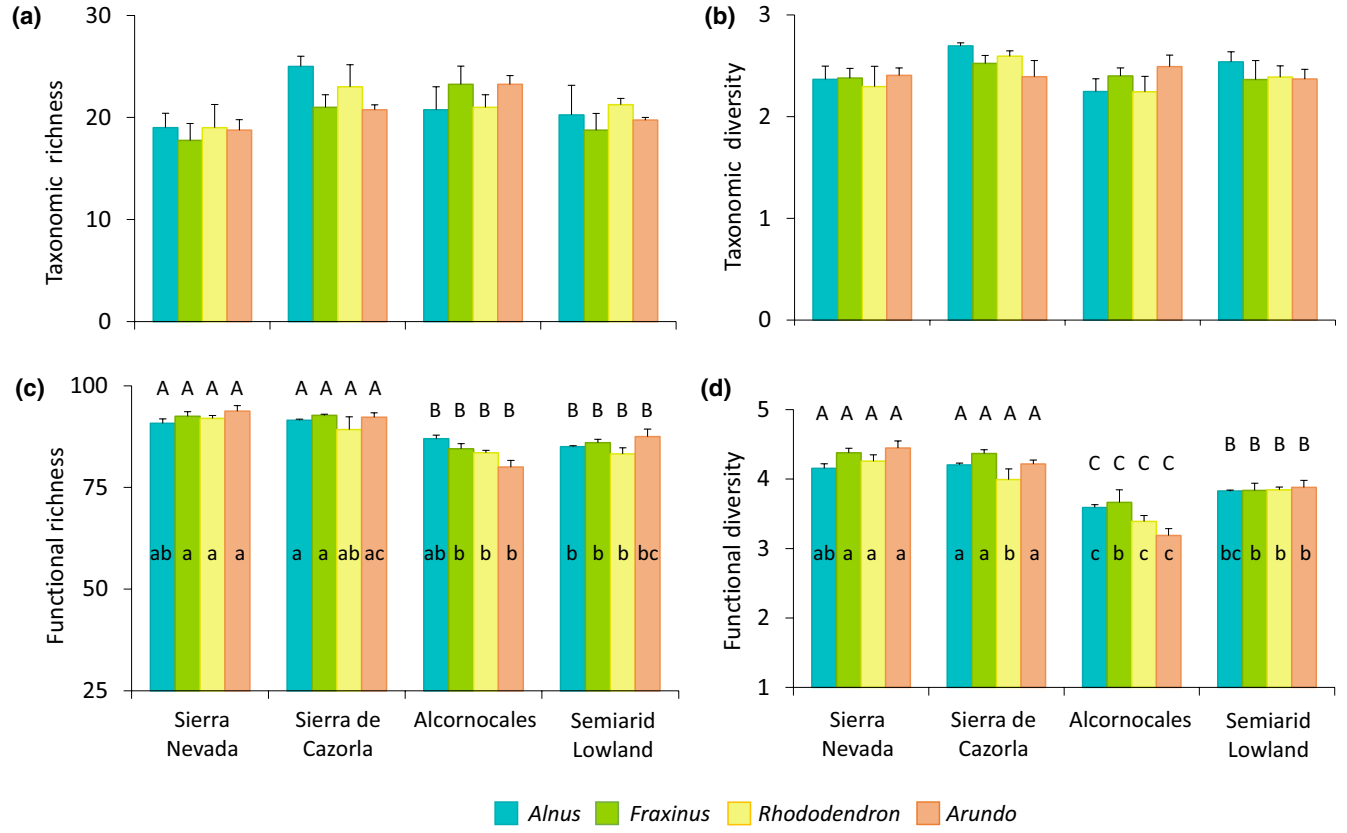

FIGURE 1 Fungal taxonomic richness (a), taxonomic Shannon diversity index (based on OUT—operational taxonomic units from DNA fingerprints) (b), functional richness (c) and functional Shannon diversity (based on CLPP: community-level physiological profiles) (d) for the four leaf-litter species (*Alnus*, *Fraxinus*, *Rhododendron* and *Arundo*) incubated in the streams of four subregions (Sierra Nevada, Sierra de Cazorla, Alcornocales, Semiarid lowland). Capital letters indicate significant differences among subregions and lowercase letters indicate intra-subregional differences among litter species (all $p < .05$)

TABLE 1 General linear model for environmental and spatial determinants of functional richness

Covariates	Estimate ± SE	*z* value	*p* value	Model pseudo-R^2
Environmental predictors				0.57
Intercept	4.479 ± 0.013	336.305	<.001	
Temperature	−0.061 ± 0.022	−2.776	.006	
NO_3^--N	0.022 ± 0.018	1.199	.231	
SRP	−0.014 ± 0.014	−0.992	.321	
EC	0.017 ± 0.019	0.898	.369	
Litter traits-RC1	0.006 ± 0.013	0.415	.678	
Litter traits-RC2	0.003 ± 0.014	0.210	.834	
Spatial predictors				0.52
Intercept	4.479 ± 0.013	336.326	<.001	
PCNM1	−0.030 ± 0.014	−2.173	.030	
PCNM2	−0.001 ± 0.014	−0.063	.950	
PCNM3	−0.020 ± 0.013	−1.516	.129	
PCNM4	−0.015 ± 0.013	−1.122	.262	
PCNM5	0.010 ± 0.014	0.749	.454	
PCNM6	0.003 ± 0.014	0.239	.811	

Abbreviations: EC, electric conductivity; PCNM, Principal Coordinates of Neighbour Matrices; RC, Rotated components of a PCA on leaf-litter traits; SRP, soluble reactive phosphorus.

taxonomic β_{Total} diversity (taxonomic-LCBD) did not include RC1 or RC2 of leaf-litter traits as predictors. Taxonomic-LCBD was positively affected by temperature and its interaction with NO_3^--N (Table 2), but negatively by the interaction between NO_3^--N and electrical conductivity (Table 2). Taxonomic LCBD was higher in the Semiarid Lowland, with the highest temperature and dissolved nutrients, than in the other subregions with high values of only one of these variables (Figure 2A). Taxonomic-LCBD measures were significantly related to PCNM1 and PCNM3, which represented broad spatial scales, and to PCNM5 representing intraregional variation within Alcornocales subregion (Figure S2). Functional-LCBD was affected positively by temperature and soluble reactive phosphorus, but negatively by conductivity, NO_3^--N and litter traits-RC1 (Table 2). However, the effects of temperature and NO_3^--N were modulated by leaf-litter traits. The negative interactions of temperature with leaf-litter traits-RC1 suggest that as litter nutrients (N and P) and relatively labile C (hemicellulose) contents increased, the positive effect of temperature on functional-LCBD decreased (Table 2, Figure 2B). The significant positive interaction of NO_3^--N with litter traits-RC1 points to a lower contribution of low-quality litter species to functional-LCBD at high NO_3^--N levels (Table 2). Functional-LCBD measures were significantly related to the PCNM1 and PCNM3, representing broad spatial scales, but also to the medium-scale variation represented by PCNM4 and PCNM5 in the warmest subregions Semiarid Lowland and Alcornocales, respectively (Figure S2).

3.6 | Relationship between fungal richness and leaf-litter decomposition

Leaf-litter decomposition (LML) expressed per degree-day had a significant positive relationship with functional richness ($F_{1,62} = 18.72$, $p < .001$, RMSE =0.54, Adj. $R^2 = 0.22$) (Figure 3A) and with functional Shannon diversity ($F_{1,62} = 22.59$, p-value <.001, RMSE =0.0004, Adjusted $R^2 = 0.26$) (Figure 3B). However, no significant relationship was found between LML and taxonomic richness or taxonomic Shannon diversity (p-value >.05 both cases).

4 | DISCUSSION

Our regional-scale field study suggested substantial similarity in taxonomic richness and Shannon diversity of aquatic fungi across subregions. Although we expected environmental factors to affect the number of fungal species (e.g. temperature: Bärlocher et al., 2008; Fernandes et al., 2009; Fernandes et al., 2012; Ferreira & Chauvet, 2011b), this was not supported by our study because equally rich and diverse communities occurred in a wide range of temperature (3.4–22.3°C) and water chemistry. Seena et al. (2019) pointed to a hump-shaped relationship between fungal taxonomic richness and a wide latitudinal gradient (3.1–26.2°C) with higher richness in temperate streams at intermediate latitudes. Our results at regional scale, which minimizes major biogeographic biases, showed a similar, but smoother, response of taxonomic richness to temperature, although differences in species richness among subregions were not significant. By contrast, we found a marked shift in fungal assemblage composition among subregions, lending support to our second hypothesis, that is, stream-water characteristics, mainly temperature, highly influences β-diversity. This was further supported by the highest values for LCBD (local contribution to β-diversity) found in streams from the Semiarid lowland and Alcornocales, the two warmest and distant subregions in our study. Despite this, the influence of riparian vegetation cannot be ruled out (Bärlocher & Graça, 2002; Ferreira et al., 2006), since species composition of riparian forest in Alcornocales appeared to be evolved from tropical-like environments (pre-Mediterranean lineages; Salinas et al., 2018), and riparian corridors in the Semiarid lowland subregion are dominated by giant graminoids (Fenoy et al., 2021; Salinas et al., 2018). However, leaf-litter species did not significantly affect fungal assemblage composition, as reported in laboratory studies based on fungal spore morphology (Bärlocher et al., 2011), strengthening the prime role of stream-water characteristics in structuring fungal species assemblages. This has been reported at regional scale (Bärlocher et al., 2011; Duarte et al., 2009; Solé et al., 2008), and at a global scale with high similarity in fungal communities among geographically distant locations with similar environmental conditions (Duarte et al., 2016; Seena et al., 2019). Our results also pointed to species replacement across subregions (i.e. net species turnover given the high similarity in richness) as the main component of β-diversity. This is in accordance with the evidence that microbial metacommunities are governed by deterministic processes across broad environmental gradients (Stegen et al., 2012; Wu et al., 2018; Zhou et al., 2014). Fungal taxonomic uniqueness of assemblages (local contribution to β-diversity, LCBD) was positively driven by temperature (and its interaction with NO^-_3-N), and negatively by electrical conductivity and NO_3^--N concentrations. However, the interactive effects of these variables suggest offsetting effects of global change drivers (temperature and NO_3^--N) on fungal community composition in stream ecosystems. While elevated temperature might boost the assemblage uniqueness by increasing β-diversity, probably by promoting the replacement of cold-stenotherm by mesophilic taxa (Seena et al., 2019), the increase in nutrient concentrations tended to homogenize fungal assemblages in streams with relatively high conductivity in our study. Increasing nutrient availability has been reported to produce stochasticity in community assembly (Chase, 2010), but its consequences on β-diversity are conflicting and apparently depend on the taxonomic metacommunity under study (Borer et al., 2014; Chase, 2010; Dunck et al., 2021). Our results agree with those of Daleo et al. (2018) in salt marshes where increased nutrient inputs enhanced the relative importance of stochastic over deterministic processes, leading to fungal community homogenization.

High functional redundancy (i.e. few species performing the same functional role as the whole community) has been reported in aquatic hyphomycete assemblages (Dang et al., 2005; Ferreira & Chauvet, 2012; Ferreira et al., 2006), suggesting that the functions ensured by these microbial decomposers would not be affected if species losses

TABLE 2 Result of beta regression analyses using environmental, leaf-litter traits and spatial variables as predictors of variation in taxonomic and functional local contribution to β-diversity (LCBD)

Covariates	Estimate ± SE	*z* value	*p* value	Model pseudo-R^2
Taxonomic β-diversity (LCBD)				
Environmental predictors				0.54
Intercept	−4.169 ± 0.009	−470.260	<.001	
Temperature	0.024 ± 0.010	2.407	.016	
NO_3^--N	−0.020 ± 0.020	−1.026	.305	
SRP	0.004 ± 0.006	0.589	.556	
EC	−0.015 ± 0.010	−1.448	.148	
Temperature × NO_3^--N	0.092 ± 0.021	4.337	<.001	
EC × NO_3^--N	−0.107 ± 0.022	−4.860	<.001	
Spatial predictors				0.49
Intercept	−4.147 ± 0.005	−754.189	<.001	
PCNM1	−0.131 ± 0.044	−2.973	.003	
PCNM2	0.056 ± 0.046	1.214	.225	
PCNM3	0.277 ± 0.044	6.338	<.001	
PCNM4	0.052 ± 0.043	1.212	.225	
PCNM5	0.120 ± 0.044	2.749	.006	
PCNM6	−0.068 ± 0.045	−1.524	.127	
Functional β-diversity				
Environmental predictors				0.71
Intercept	−4.238 ± 0.038	−111.831	<.001	
Temperature	0.611 ± 0.059	10.327	<.001	
NO_3^--N	−0.356 ± 0.050	−7.123	<.001	
SRP	0.138 ± 0.040	3.417	.001	
EC	−0.170 ± 0.041	−4.099	<.001	
Litter traits-RC1	−0.114 ± 0.037	−3.059	.002	
Temperature × Litter traits-RC1	−0.190 ± 0.052	−3.663	<.001	
NO_3^--N × Litter traits-RC1	0.165 ± 0.052	3.170	.002	
Spatial predictors				0.60
Intercept	−4.216 ± 0.04521	−93.260	<.001	
PCNM1	0.301 ± 0.03804	7.917	<.001	
PCNM2	0.010 ± 0.03745	0.268	.789	
PCNM3	0.214 ± 0.04839	4.433	<.001	
PCNM4	0.118 ± 0.04126	2.871	.004	
PCNM5	−0.090 ± 0.03656	−2.469	.014	
PCNM6	0.025 ± 0.03192	0.775	.439	

Abbreviations: EC, electric conductivity; PCNM, Principal Coordinates of Neighbour Matrices; RC, Rotated components of a PCA on leaf-litter traits; SRP, soluble reactive phosphorus.

occur within the assemblages. While subregions did not differ in species richness, fungal communities were functionally richer in the colder subregions than the warmer ones. Recent studies highlight the critical role that microbial dormancy and its environmental determinants can exert on B-EF relationships (Kearns et al., 2016). Because we assessed taxonomic diversity as number of OTUs based on DNA fingerprinting, we cannot rule out the hypothesis that increased temperature would have increased the proportion of dormant fungal taxa, thus rendering lower functional richness. This is supported by microbial culture data indicating that higher temperature increases the costs of sustaining life, thus fostering inactivation (Wörmer et al., 2019).

Our results strongly suggest that temperature boosted functional heterogeneity among fungal assemblages, while water chemistry (i.e. conductivity and NO_3^--N concentration) had a homogenizing effect. This indicates that changes in functionality are connected with environmental species sorting, and not just with environmental effects

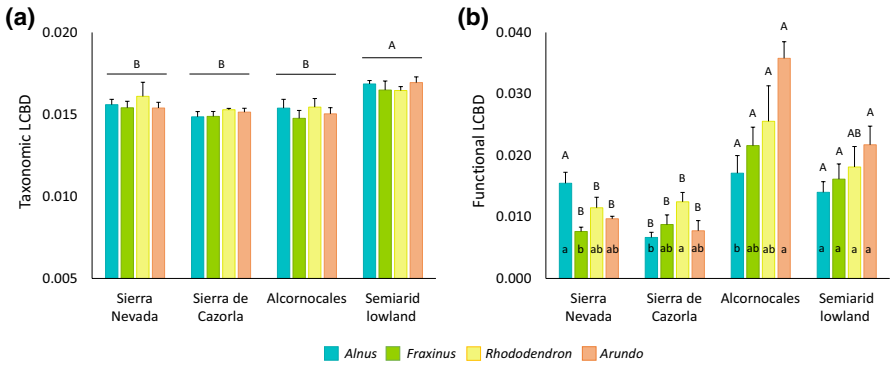

FIGURE 2 Local contribution to (a) taxonomic and (b) functional β-diversity (LCBD) of samples. for the four leaf-litter species (*Alnus*, *Fraxinus*, *Rhododendron* and *Arundo*) incubated in the streams of four subregions (Sierra Nevada, Sierra de Cazorla, Alcornocales, Semiarid lowland). Capital letters indicate significant differences among subregions and lowercase letters indicate intra-subregional differences among litter species (all $p < .05$)

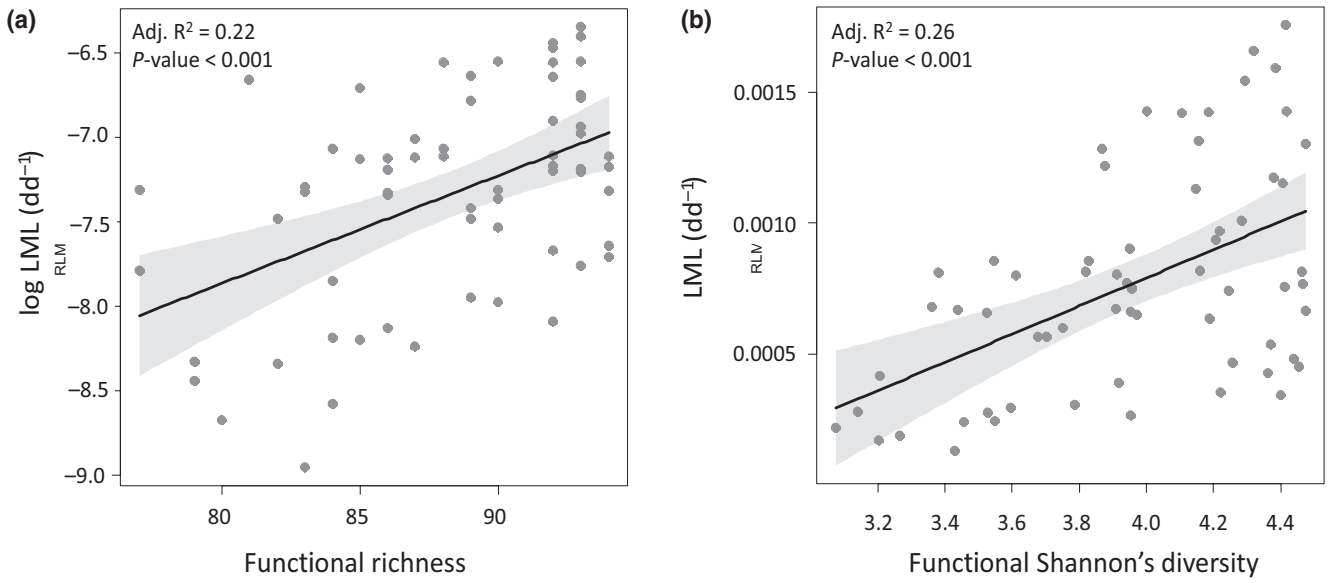

FIGURE 3 Relationship between leaf decomposition (LML) and functional richness (a) or functional Shannon diversity (b) across all four subregions (Sierra Nevada, Sierra de Cazorla, Alcornocales, Semiarid lowland). $n = 64$. dd^{-1}, per degree-day

on the functional expression of a given fungal taxon. Despite this connection, however, total functional β-diversity was much lower (4-fold) than the taxonomic β-diversity, which most likely indicates the stronger environmental filtering on fungal species compared to the functions. Most functions related to carbon acquisition are shared across fungal assemblages. Our results also pointed to a complex interplay of factors on functional variability (LCBD-functional) because of the observed effects of soluble reactive phosphorus and the interactions of temperature or NO_3^--N with leaf-litter chemistry. The significant relationship between large-scale spatial predictors (i.e. PCNM 1 and PCNM 3) and LCBD suggests that LCBD is spatially structured across streams and might represent missed patterns of spatial environmental conditions among subregions. The higher heterogeneity in Alcornocales and Semiarid Lowland might explain the strong correlation between medium-scale spatial descriptors and taxonomic or functional LCBD.

Our third hypothesis, postulating that functionality might be more steady than community structure, was supported since functional β_{Total} was lower than taxonomic β_{Total}. In stream ecosystems, fungal B-EF relationships have been mostly evaluated by examining the effects of species number on leaf-litter decomposition, often concluding high functional redundancy among fungal species (Bärlocher & Graça, 2002; Gonçalves et al., 2015; Pascoal et al., 2005). In soils, fungal species richness had a positive effect on carbon cycling in low-diversity (≤10 taxa) experiments, but this relationship was less frequent in high-diversity (>10 taxa) experiments (Nielsen et al., 2011). In streams, there is no information on the critical threshold of fungal taxonomic richness below which ecological functions become compromised. However, in our study, fungal taxonomic richness was high and similar across the subregions (>18 OTUs in all samples), making it difficult to test for potential functional redundancy exists among communities.

However, our study provided evidence that fungal communities with similar taxonomic richness can differ in functionality, probably with fungal communities reaching relatively high functional redundancy but with differences in functional richness. So, why do fungal communities invest more in functional richness in cold waters than in warm waters? When temperature increases, respiratory costs may overcome the benefits of enzyme production, and microbes would switch the carbon allocation from growth to respiration, that is, reducing carbon use efficiency (Allison, 2014). So, it is conceivable that fungal communities have to adjust metabolic traits to offset declines in carbon use efficiency at higher temperatures. However, whether this response is a consequence of environmental selection of genotypes or is due to phenotypic plasticity of aquatic fungi remains unknown. Thus, under a climate change scenario, high taxonomic diversity might not guarantee ecosystem functioning (Parain et al., 2019) since more species would be required to maintain it under thermal stress (García et al., 2018). Fungal diversity increases the nutritional value and palatability of plant litter for consumers (Canhoto & Graça, 2008; Jabiol et al., 2013). Thus, under a warming scenario, faster decomposition would facilitate rapid, but inefficient, uptake of carbon by microorganisms, reducing carbon assimilation by invertebrates, with negative consequences for the global decomposition process (Marks, 2019; Siders et al., 2018).

The space-for-time (SFT) substitution approach, using different spatial scales, is increasingly being applied in biodiversity modelling to project warming-driven changes in species diversity (Blois et al., 2013; Guisan & Thuiller, 2005). The SFT substitution approach allowed us to test B-EF hypotheses at a regional scale with a wide thermal gradient. We are aware that a pure SFT substitution approach provides a snapshot view of ecosystems. Therefore, once the target patterns have been identified, the combination of spatial variation with time-series data (Damgaard, 2019) may provide deeper knowledge on the responses of communities and processes to warming.

In our study, functional richness was positively correlated with leaf-litter decomposition, supporting our fourth hypothesis. Higher microbial decomposition of leaf litter has been reported under relatively cold conditions or at higher elevation (Pérez et al., 2018; Taylor & Chauvet, 2014), indicating greater carbon use efficiency in the cold subregions than in the warm ones. This was likely due to the lower metabolic stress, which allows maintaining higher functional richness in the cold subregions. Thus, it seems that higher biodiversity (and species complementarity) might be required to sustain functionality and decomposition efficiency at higher temperatures. While our study does not grant these mechanisms, our findings highlight the need of gaining more in-depth knowledge about the effects of shifting environmental conditions on microbial functionality in stream ecosystems.

In conclusion, our results suggest that the study of B-EF relationship of aquatic fungi is not easily approached under field conditions exclusively through taxonomic richness analysis, since assemblages similarly rich in taxa occurred across ample environmental gradients. Instead, changes in taxon composition (β-diversity) and functional diversity appear to be key for understanding how B-EF relationships are modulated by temperature and water chemistry. Moreover, our results based on functional richness suggest that under the umbrella of functional redundancy, there are hidden metabolic responses of fungal community that have been scarcely explored. We found significant effects of the environmental variables, mainly the temperature, on fungal functionality, suggesting that the reduction in functional diversity by increased temperature may lower the decomposition efficiency. However, other functionality measures (e.g. fungal reproduction and fungal biomass production) may show different response patterns. Thus, future studies should consider biodiversity and multifunctionality relationships to improve our understanding of the effects of global change on ecosystem functioning.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Conceptualization: JJC and EF; Investigation: EF, AP, JRR, DB, FJML, JJC, CP and FC; Formal analysis: EF and AP; Writing—original draft: EF; Writing – review & editing: JJC, AP, CP and FC; Funding acquisition: JJC, CP and FC.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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