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Fungal–fungal and fungal–bacterial interactions in aquatic decomposer communities: bacteria promote fungal diversity

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Abstract. Fungi produce a variety of extracellular enzymes, making recalcitrant substrates bioavailable. Thus, fungi are central for the decomposition of dead organic matter such as leaf litter. Despite their ecological importance, our understanding of relationships between fungal species diversity and ecosystem functioning is limited, especially with regard to aquatic habitats. Moreover, fungal interactions with other groups of microorganisms such as bacteria are rarely investigated. This lack of information may be attributed to methodological limitations in tracking the biomass of individual fungal species in communities, impeding a detailed assessment of deviations from the overall performance expected from the sum of individual species' performances, so-called net diversity effects (NDEs). We used fungal species-specific biomolecular tools to target fungal–fungal and fungal–bacterial interactions on submerged leaves using four cosmopolitan aquatic fungal species and a stream microbial community dominated by bacteria. In microcosms, we experimentally manipulated fungal diversity and bacterial absence/presence and assessed functional performances and fungal community composition after 14 d of incubation. Fungal community data were used to evaluate NDEs on leaf colonization. The individual fungal species were functionally distinct and fungal cultures were on average more efficient than the bacterial culture. In absence of bacteria, NDEs correlated with growth rate (negatively) and genetic divergence (positively), but were predominantly negative, suggesting that higher fungal diversity led to a lower colonization success (niche overlap). In both absence and presence of bacteria, the overall functional performances of the communities were largely defined by their composition (i.e., no interactions at the functional level). In the presence of bacteria, NDEs correlated with genetic divergence (positively) and were largely positive, suggesting higher fungal diversity stimulated colonization (niche complementarity). This stimulation may be driven by a bacteria-induced inhibition of fungal growth, alleviating competition among fungi. Resulting feedback loops eventually promote fungal coexistence and synergistic interactions. Nonetheless, overall functional performances are reduced compared to bacteria-free cultures. These findings highlight the necessity to conduct future studies, investigating biodiversity–ecosystem functioning relationships using artificial systems, without exclusion of key organisms naturally co-occurring in the compartment of interest. Otherwise, study outcomes might not reflect true ecological relationships and ultimately misguide conservation strategies.

Key words: antagonism; biodiversity–ecosystem functioning; cellulolytic enzymes; community composition; complementarity; diversity effects; fungal interactions; fungal–bacterial interactions; ligninolytic enzymes; microbial decomposers; saprotrophs; traits.

Introduction

The decomposition and recycling of leaf litter is a key process in ecosystems, which is realized by microbial (bacteria and fungi) and invertebrate decomposers. In particular fungi play a pivotal role in this process (Dighton and White 2017). Using a repertory of extracellular enzymes, they have the capability of converting a

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broad range of non-utilizable low- (e.g., phosphate esters and oligosaccharides) and high-molecular-weight organic compounds (e.g., cellulose, hemicellulose, and lignin) to bioavailable substances (e.g., inorganic nutrients or monomeric carbohydrates [Evans and Hedger 2001, Kubicek and Druzhinina 2007]). Yet, our (mechanistic) understanding of the relationship between fungal community structure (e.g., diversity and composition) and ecosystem functioning (i.e., biodiversity–ecosystem functioning; B-EF) is still at the beginning (De Laender et al. 2016, Grossart and Rojas-Jimenez 2016). Consequently, impacts of human-induced biodiversity loss on ecosystem-level processes, such as leaf litter decomposition, remain difficult to predict (Bell et al. 2009, Canhoto et al. 2016, Wright et al. 2017).

In aquatic and terrestrial ecosystems, there is vast potential for positive diversity effects on fungal-mediated leaf litter decomposition, which is considered to be largely based on resource partitioning (Gessner et al. 2010). In fungal assemblages, resource partitioning is realized via complementary extracellular enzyme inventories and varying activity patterns (i.e., functional divergence; Gessner et al. 2010). The extent of complementarity and thus decomposition efficiency is assumed to increase with increasing genetic divergence (Eichlerová et al. 2015, Andrade et al. 2016). Because of differences in biotic as well as abiotic drivers of diversity, abundance, and activity, however, the relative importance of mechanisms underlying B-EF relationships can vary between aquatic and terrestrial habitats (Gessner et al. 2010, Bärlocher and Boddy 2016). Because of a higher degree of functional redundancy, the scope for complementarity is assumed to be much lower in aquatic compared to terrestrial leaf decomposing fungal communities (Bell et al. 2009, Gessner et al. 2010, Gonçalves et al. 2015). It is instead assumed that competitive and productive species dominate leaf decomposition in aquatic ecosystems (i.e., dominance or selection effect [Bärlocher and Corkum 2003, Ferreira and Chauvet 2012, Gonçalves et al. 2015]). Yet, the relative importance of complementarity and dominance effects for fungal B-EF relationships as well as the fundamental mechanisms supporting fungal species co-occurrence in aquatic ecosystems are poorly understood (Bärlocher and Corkum 2003, Krauss et al. 2011, Bärlocher 2016). Most findings on interactions in aquatic fungi were generated under controlled conditions, excluding other groups of organisms, such as bacteria, which, together with fungi, dominate leaf decomposition in aquatic habitats (Hieber and Gessner 2002). Therefore, and as these organism groups inevitably co-occur and closely interact with each other (Baschien et al. 2009, Deveau et al. 2018), the universality of findings on fungal interactions, generated under restrictive laboratory conditions, is questionable.

A key to investigating fungal B-EF relationships is the assessment of net diversity effects (NDEs). NDEs are measured as the deviation between observed productivity in multispecies assemblages and productivity predicted from individual species, weighted by their initial contribution to an assemblage, under the null hypothesis that there are no interactions between species (Loreau and Hector 2001). Assessing the mechanisms underlying NDEs (e.g., complementarity or dominance), however, necessitates tracking the biomass of individual species in complex assemblages not only at the initial but also at later successional stages (Bärlocher and Corkum 2003, Duarte et al. 2006, Grossart and Rojas-Jimenez 2016). This has just recently been enabled via TaqMan® quantitative real-time PCR (qPCR) assays specific to widespread aquatic fungal species (Baudy et al. 2019).

In order to address current knowledge gaps, we conducted a multifactorial laboratory microcosm experiment to assess fungal–fungal and fungal–bacterial interactions on submerged leaves in the absence and presence of stream bacteria. As model organisms, four widely distributed aquatic fungi—belonging to a polyphyletic fungal group adapted to submerged leaf litter (so called aquatic hyphomycetes)—and a natural community of stream bacteria co-occurring with aquatic hyphomycetes in the wild, were used. We experimentally manipulated fungal diversity and bacterial absence/presence and analyzed functional variables (i.e., leaf mass loss, total fungal biomass, and enzyme activities) and community composition after 14 d of incubation.

We expected fungal (bacteria-free) monocultures to show varying functional performances (i.e., different functional traits; sensu Aguilar-Trigueros et al. [2015]). Furthermore, we hypothesized that fungal–fungal interactions in mixed cultures would be species-dependent and would change from antagonistic (competitive) to synergistic (complementary) with increasing functional or genetic diversity of the component species (Andrade et al. 2016). Lastly, we anticipated fungal–bacterial interactions to be species dependent, but antagonistic in nature (Mille-Lindblom and Tranvik 2003, Romaní et al. 2006), resulting in negative NDEs on the investigated variables.

Materials and Methods

Microorganisms and leaf substrate

Strains of the aquatic hyphomycete species *Alatospora acuminata* (DSM 104360), *Heliscella stellata* (DSM 104386), *Neonectria lugdunensis* (DSM 104361), and *Tetracladium marchalianum* (DSM 104373) were selected as model fungi. This selection covers ascomycete species frequently detected in aquatic decomposer communities of the temperate latitudes and exhibits a gradient of sensitivities to anthropogenic stress (e.g., Solé et al. [2008], Fernández et al. [2016]). The strains were isolated from German streams and deposited in the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany [Baudy et al. 2019]). In the laboratory, isolated strains were exclusively

handled under sterile conditions. Axenic cultures of the fungi were grown on 15 mL of malt extract agar (10 g malt extract/L, 20 g agar/L) in Petri dishes at 16°C in darkness for 21 d. Cylindrical agar plugs (5 mm diameter) were cut from the growing edges of the colonies—to sample young, metabolically active mycelium—serving as fungal inoculum of leaf disks in microcosms.

The stream inoculum, harboring the bacterial community considered as a factor (absence or presence) in this study, originated from the near-natural stream Hainbach, Germany (49.24202° N; 8.04586° E). Therefore, stream water and submerged leaf litter were collected in a pre-autoclaved 1-L Schott glass bottle, a day before the start of the experiment, in December 2017. In the laboratory, using a sterilized filtration apparatus, the stream water was passed through glass fiber filters (1.2 μm nominal pore size; GF/C; Whatman GmbH, Dassel, Germany) to obtain approximately 500 mL of stream inoculum (for details see Danger et al. [2013]). A 250-mL aliquot was sterilized for use in bacteria-free treatments ensuring equivalent nutrient conditions across all treatments. Sterilization was realized by filtration through a polycarbonate filter (0.2 μm nominal pore size; Isopore™; Merck KGaA, Darmstadt, Germany) to remove microbial biomass, and autoclavation to halt any microbial activity. Both stream inoculum and sterilized stream water were kept in pre-autoclaved 250-mL Schott glass bottles at 4°C under permanent stirring until use in the experiment. It was presumed that the size-fractionated stream inoculum (i.e., ≤1.2 μm) excludes larger eukaryotic microorganisms (Morris and Nunn 2013) and is dominated by a taxonomically and functionally diverse bacterial community (Leflaive et al. 2008), whereas other unicellular organisms smaller than 1.2 μm, such as archaea, only contribute a minor fraction to the microbial biomass in the inoculum (e.g., Manerkar et al. [2008], Uyaguari-Diaz et al. [2016], Lambirth et al. [2018]). Therefore, the stream inoculum will in the following be referred to as "bacterial inoculum" and associated observations are solely discussed with respect to bacterial activity as done elsewhere (cf. Mille-Lindblom et al. [2006], Danger et al. [2013]).

Leaves of *Alnus glutinosa* (L.) Gaertn. (black alder)—a widespread European riparian tree species (Copolovici et al. 2014)—were used as model substrate. Only leaves with no visible signs of damage or symptoms of disease were handpicked from trees near Landau, Germany (49.20116° N; 8.09331° E) shortly before abscission in 2016 and stored at −20°C until further use (for ~1 yr). After thawing, disks (16 mm diameter) were cut from the leaves, avoiding the midvein. Leaf disks were subsequently processed in packs of 20. To account for variability associated with initial mass losses due to physical leaching of soluble leaf components (Petersen and Cummins 1974), leaf disks were leached for 48 h in 50 mL of nutrient medium (adjusted to pH 7; for composition see Dang et al. [2005]). Subsequently, leaf disks were dried at 60°C for 24 h and their initial dry weight was determined to the nearest 0.01 mg. On the day prior to the experiment, each test vessel—a 100-mL Erlenmeyer flask—was provided with a pre-weighed leaf disk pack, covered with aluminum foil, autoclaved, and kept overnight in a sealed ethanol-disinfected box. At the beginning of the experiment, nine leaf disk packs were directly sampled, dried at 60°C for 24 h, and weighed again to quantify leaf mass losses related to the autoclaving procedure.

Experimental design

The microcosm experiment followed a 12 × 2 factorial design. Twelve fungal treatments, comprising no fungi, four monocultures, six binary, and one quaternary combination of fungal species, were cultivated in absence and presence of bacteria. Each treatment was replicated six times ($n = 6$), which resulted in a total number of 144 microcosms ($N = 144$).

The microcosms were prepared under sterile conditions in a laminar flow cabinet (UV-treated for 30 min prior use; NU-437-500E; Nuaire, Plymouth, MN, USA) using autoclaved materials. A microcosm consisted of a 100-mL Erlenmeyer flask containing 20 leaf disks (see Microorganisms and leaf substrate), 60 mL of nutrient medium, four agar plugs, and 1 mL of bacterial inoculum or sterile stream water, respectively. Procedures for fungal and bacterial inoculation followed the methods described in Andrade et al. (2016) and Danger et al. (2013), respectively. Microcosms designated to contain one, two, or four fungal species were equipped with four, two, or one cultivated agar plug(s) per species, respectively, while microcosms with no fungi were equipped with four sterile agar plugs. This procedure followed the assumption that one colonized agar plug already constitutes an excess of fungal inoculum, by which the leaf colonization rate of each species is maximized (cf. Bärlocher and Corkum [2003]). Although initial microbial biomass was not further characterized, the setup of microcosms containing fungal and bacterial inoculum likely resulted in a high fungal/bacterial biomass ratio (≫1), which is typical for decomposing leaves throughout various successional stages (Findlay et al. 2002, Hieber and Gessner 2002, Manerkar et al. 2008). All microcosms were sealed with cellulose plugs, randomly distributed on a horizontal shaker (model VKS 75 B control; Edmund Bühler GmbH, Bodelshausen, Germany) set at 110 rpm, and incubated at 16°C in darkness. To minimize location effects all microcosms were shuffled daily. After 7 d, the nutrient medium was renewed; after 14 d, the experiment was terminated and the leaf disks were recovered. Per microcosm, one leaf disk was preserved in a capped 12-mL round-bottom plastic centrifuge tube and stored at −20°C for later enzyme activity analyses. Two leaf disks were conserved in 2% formaldehyde/0.1% sodium pyrophosphate solution and stored at 4°C for analysis of bacterial densities, and a further two leaf disks were preserved for potential future analysis. The remaining leaf disks were preserved

in pre-weighed 2-mL Eppendorf tubes, frozen (−20°C) before freeze drying for 24 h, weighed to the nearest 0.01 mg, and stored at −20°C for later ergosterol and deoxyribonucleic acid (DNA) analysis. Two and one replicates of the binary cultures *A. acuminata–H. stellata* ($n = 4$ remaining) and *H. stellata–N. lugdunensis* ($n = 5$ remaining), respectively, were contaminated with waste medium of various cultures during the medium renewal and were therefore excluded from further analyses ($N = 141$ remaining).

Ergosterol analysis

Total fungal biomass was analyzed as the amount of the fungal membrane molecule ergosterol, following the procedure described in Gessner (2005). Ergosterol was extracted from 30 to 50 mg of lyophilized leaf material, weighed to the nearest 0.01 mg, in 10 mL of alkaline methanol at 80°C. Solubilized ergosterol was purified via solid phase extraction (Sep-Pak® Vac RC tC18 500 mg sorbent; Waters, Milford, MA, USA), eluted in isopropanol, and stored at −20°C until analysis. After reaching room temperature, the extracts were quantified for ergosterol using a high-performance liquid chromatography (HPLC) system (1200 Series; Agilent Technologies, Santa Clara, CA, USA) as detailed in Zubrod et al. (2011). One sample of the *H. stellata* monoculture vaporized during ergosterol extraction and could therefore not be used for further analyses ($n = 5$).

Enzyme activity assays

Using artificial substrates, activities of hydrolases and oxidases were analyzed fluorometrically and colorimetrically, respectively, as described in DeForest (2009) modified for leaf samples. The assessed degradative enzymes were phosphatases (PHO; EC 3.1.3.1 and 3.1.3.2; targeting phosphate esters), α-1,4-glucosidase (AGL; EC 3.2.1.20; targeting starch and maltose), β-1,4-glucosidase (BGL; EC 3.2.1.21; targeting cellulose), cellobiohydrolase (CEL; EC 3.2.1.91; targeting cellulose), β-1,4-xylosidase (XYL; EC 3.2.1.37; targeting hemicellulose), peroxidase (PER; EC 1.11.1.7; targeting lignin), and phenol oxidase (PHE; EC 1.10.3.2; targeting lignin). Leaf disks were thawed and homogenized in 350 mL of nutrient medium. Hydrolase and oxidase activities in sample homogenates were analyzed in 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) using a microplate reader (Infinite 200; Tecan Group, Männedorf, Switzerland). All sample preparation and analysis steps were performed in a temperature-controlled laboratory room at approximately 18°C. For a detailed description of used artificial substrates, assay procedures, dispensing volumes, plate setups, and fluorescence/absorbance calculations, see Appendix S1. Ultimately, 13 out of 987 enzyme activity measurements apparently resulted from handling errors during sample processing and were thus excluded from further analyses. This concerned single replicates of the bacteria-free cultures *A. acuminata* (PER), *T. marchalianum* (BGL and XYL), *A. acuminata–N. lugdunensis* (PHE), *A. acuminata–T. marchalianum* (CEL and PHO), and in the control (PER and PHE) as well as the bacteria-containing cultures *H. stellata* (CEL), *N. lugdunensis* (CEL), *A. acuminata–H. stellata* (PHE), *H. stellata–N. lugdunensis* (PHE), and all fungi (XYL).

Fungal species–specific quantitative real-time polymerase chain reaction (qPCR)

Individual biomasses of the four model fungi were estimated via DNA concentrations obtained from species-specific qPCR analyses, following Baudy et al. (2019). DNA was extracted from 10 to 20 mg of lyophilized leaf material, weighed to the nearest 0.01 mg, using the FastDNA® Spin Kit for Soil in conjunction with the FastPrep™-24 5G Instrument (MP Biomedicals Germany GmbH, Eschwege, Germany) as per manufacturer's instructions. Ten times diluted extracts were used to quantify DNA of the model fungi in species-specific TaqMan® qPCR reactions (Applied Biosystems, Waltham, MA, USA) performed in a Mastercycler® ep gradient S (Eppendorf, Hamburg, Germany). For a detailed description of the used species-specific TaqMan® assays, reaction compositions, cycling conditions, and data analysis see Baudy et al. (2019).

Bacterial densities

Following the procedure described in Buesing (2005), bacterial densities were analyzed in all replicates, which received bacterial inoculum. Bacteria were detached from the leaf substrate via ultrasonication, filtered onto an inorganic filter membrane (0.2 μm nominal pore size; Anodisk™; Whatman GmbH, Dassel, Germany), and stained using the fluorophore SYBRGreen II (Molecular Probes, Eugene, OR, USA). Digital pictures of filter sections were captured with an epifluorescence microscope (Axio Scope.A1; Carl Zeiss, Jena, Germany) using a 1,000× magnification. Afterwards, following the procedure described in Zubrod et al. (2011), cells were systematically counted by image analysis software (Axio Vision 4.8.2; Carl Zeiss, Jena, Germany).

General calculations

Leaf mass loss (L) was expressed in percent and calculated as follows:

$$L = \frac{(w_i \times 0.924 - w_f \times 1.333)}{(w_i \times 0.924)} \times 100,$$

where w_i and w_f refer to the initial and final dry weight of 20 and 15 leaf disks, respectively, and 0.924 and 1.333 are empirical factors controlling for leaf mass losses due to the autoclaving procedure and the destructive sampling of five leaf disks, respectively. Ergosterol and DNA

concentrations were expressed as μg/g leaf dry weight and were calculated as described in Baudy et al. (2019). Fungal growth rates were calculated from ergosterol concentrations in bacteria-free fungal monocultures and were expressed as μg ergosterol/g leaf dry weight/d. Enzyme activities were expressed as μmol/(h × g) leaf dry weight and calculated as described in Appendix S1: Eqs. S1–S6. All data were normalized to the control (i.e., no fungi and no bacteria present) and any resulting negative values were replaced by zero (concerning 115 out of 987 enzyme activity measurements).

Prediction of functional performances

Biomass-specific functional performances (B-SFPs) were calculated from fungal monocultures in absence of bacteria and from the bacteria-only treatment, by normalizing overall functional performances (OFPs) to ergosterol, DNA, or bacterial cell concentrations. In order to characterize functional traits of the fungi, B-SFPs were based on ergosterol (Appendix S2: Table S1). Compared to DNA, ergosterol concentrations show less interspecific variability (Baudy et al. 2019), which is why B-SFPs based on this biomarker are more readily comparable among species. To predict OFPs in mixed cultures following the assumption that biomasses of component species are not equal, B-SFPs were based on individual fungal species' DNA concentrations and bacterial cell densities. These predictions were calculated as the sum of B-SFPs of the component fungi and bacteria (Appendix S2: Table S2) multiplied by species-specific DNA concentrations and bacterial cell densities measured in mixed cultures. Observed and predicted OFPs were tested against the null hypothesis of additivity (Bärlocher and Corkum 2003).

Calculation of genetic divergences

The mean genetic divergence of fungal strains in mixed cultures served—besides species number—as a measure of diversity and was based on the internal transcribed spacer (ITS) region of the ribosomal DNA operon—the most frequently used barcoding region for fungi (Schoch et al. 2012). ITS sequences of the model fungi (GenBank accession numbers MH930815, MH930820, MH930822, and MH930823) were trimmed to a common region (i.e., partial ITS1, 5.8S, and ITS2) and aligned, whereupon genetic divergences for combinations of two and four species were calculated as pairwise distances and overall mean distance, respectively, using the bioinformatics software MEGA X (Kumar et al. 2018) with default settings (maximum composite likelihood substitution model).

Calculation of diversity effects on leaf colonization

To assess interactions between co-occurring fungi on leaf colonization, which requires information on individual species' biomasses, NDEs based on species-specific DNA concentrations in monocultures and mixed cultures were calculated applying a modified Price equation (i.e., tripartite partition [Fox 2005]). Based on the assumption that the fungi perform in mixed cultures equally well as in monocultures (i.e., additivity), NDEs on DNA concentrations (ΔY) were calculated as

$$\begin{aligned}\Delta Y = {} & NE_{uw}(M)E_{uw}(\Delta RY) \\ & + N\,\mathrm{Cov}_{uw}\left(M, \frac{RY_O}{RYT_O} - RY_E\right) \\ & + N\,\mathrm{Cov}_{uw}\left(M, RY_O - \frac{RY_O}{RYT_O}\right),\end{aligned}$$

which allows discrimination between trait-independent complementarity (expectation term), dominance (first covariance term), and trait-dependent complementarity effects (second covariance term; for a detailed description of these effects, see Fox [2005]). N is the total number of fungal species in a mixture (i.e., 2 or 4), E_{uw} is the unweighted mean of the N species in a mixture, and M is the DNA concentration of each species in monoculture. RY_O is the observed relative DNA concentration of a species in a mixture (i.e., its observed DNA concentration in the mixture divided by M) and RY_E is the expected relative DNA concentration of a species in a mixture, which is its proportion to the inoculum (i.e., 0.5 or 0.25 for binary or quaternary species combinations, respectively). ΔRY is the deviation from expected relative DNA concentrations of a species in a mixture (i.e., the difference between RY_O and RY_E) and RYT_O is the total observed relative DNA concentration of a mixture (i.e., the sum of all species' RY_O in a mixture).

NDEs are an indicator of general synergistic (positive values) or antagonistic interactions (negative values). Trait-independent complementarity effects are indicative of interactions resulting in a mutual benefit (e.g., niche partitioning; positive values) or in a mutual expense (e.g., niche overlap; negative values). Dominance effects are indicative of rapidly growing species dominating at the expense of slowly growing species (positive values) or slowly growing species dominating at the expense of rapidly growing species (negative values), while in both cases species occupy similar niches. Trait-dependent complementarity effects are indicative of interactions benefiting rapidly (positive values) or slowly growing species (negative values), but in both cases not at the expense of other species (Fox 2005).

Statistical analyses

Prior to statistical testing, extreme values were identified by visual inspection of box plots (values deviating from the box by more than three times the interquartile range) and excluded from further analyses if values were identified at both the treatment level ($n = 6$) and the variable level ($N = 141$). Accordingly, one replicate of the *H. stellata–T. marchalianum* culture treatment was

excluded from analysis of NDEs. Normality and homogeneity of variances was tested using the Shapiro-Wilk and Levene's test, respectively.

To assess the effects of fungal diversity, species combination (nested within diversity) and bacterial presence as well as their interactions on response variables, three-way nested analysis of variance (ANOVA) was performed on the original data if the presumptions for parametric testing were met. Otherwise ANOVAs were performed on square-root-transformed or rank-transformed data (Conover and Iman [1981]; but see also Seaman et al. [1994] for critique). To assess whether B-SFPs based on ergosterol concentrations represent ecologically meaningful functional traits (i.e., significantly different from zero) of individual fungi, one-sample *t*-tests and Wilcoxon signed-rank tests (as nonparametric alternative) were performed. Single comparisons between observations and predictions and between observations in bacterial presence and absence were performed using paired and unpaired two-sample tests, respectively (i.e., *t*-tests and Wilcoxon rank-sum tests as nonparametric alternative). Multiple comparisons between DNA concentrations of fungal mono- and mixed cultures were performed using ANOVAs followed by Dunnett's tests, or if the presumptions for parametric testing were not met, by Wilcoxon rank-sum tests with Bonferroni correction for multiple comparisons. To assess associations between two variables, Pearson's correlation and Spearman's rank correlation (as nonparametric alternative) were used.

Multivariate data were $\log(x + 1)$-transformed and min–max normalized to decrease the discriminatory power of enzymes with high activities and to enable the simultaneous analysis of different types of variables (i.e., leaf mass loss, growth rates, and enzyme activities). Replicates with missing values were excluded from multivariate analysis. To assess the effects of fungal diversity, species combination (nested within diversity) and bacterial presence as well as their interactions on enzyme activity profiles, permutational multivariate analysis of variance (PERMANOVA) was performed on enzyme activity profiles of all cultures. Functional dissimilarities between fungal species (i.e., in bacteria-free monocultures) were calculated from biomass-specific functional performances (i.e., leaf mass loss, enzyme activities, and growth rates, based on ergosterol concentrations) using Bray–Curtis dissimilarity. For the visualization of dissimilarities of the enzyme activity profiles as well as species-specific functional performances, data were displayed via non-metric multidimensional scaling (NMDS) using Bray–Curtis dissimilarity. All statistics and figures were prepared using R version 3.5.2 (R Development Core Team 2018) as well as the add-on packages "multcomp", "plotrix", and "vegan". The term "significant(ly)" is exclusively used with regard to statistical significance throughout this study.

Results

Leaf mass loss

Generally, leaf decomposition efficiency was shaped by complex interactions between fungal species number, fungal species identity, and bacterial presence (Fig. 1a; Appendix S2: Table S3). The model fungi differed in their degradative capabilities with mean leaf mass losses ranging between 0.5 and 21.0% (Fig. 1a), and biomass-specific leaf mass losses were nearly identical for *A. acuminata*, *N. lugdunensis*, and *T. marchalianum* (Appendix S2: Table S1). Nonetheless, changes in biomass-specific leaf mass loss significantly contributed to the functional separation of the species (Fig. 2a). Observed leaf mass losses in bacteria-free fungal mixed cultures (6.4%–22.4%) did hardly exceed the performance of the most productive fungal monoculture (i.e., *T. marchalianum*: 21.0%) and largely matched predictions (Fig. 1a). Only in the quaternary species combination observations were significantly higher than predictions, indicating ecologically meaningful interactions (Fig. 1a). The bacteria-only culture was capable of degrading a significant amount of leaf mass (4.2%) but was 2.8 times less productive than the average of all fungal monocultures (11.6%; Fig. 1a). Leaf mass loss in bacteria-containing fungal cultures was generally lower than in bacteria-free ones, except in the monocultures of the two least productive fungi *A. acuminata* and *H. stellata* (Fig. 1a). Observed leaf mass loss in bacteria-containing fungal mono- and mixed cultures in large part matched predictions (Fig. 1a). Observations only deviated significantly from predictions in the bacteria-containing fungal monocultures *N. lugdunensis* (overpredicted) and *T. marchalianum* (underpredicted) and the binary culture *A. acuminata–N. lugdunensis* (overpredicted), indicating ecologically meaningful interactions (Fig. 1a).

Ergosterol

Ergosterol concentrations (i.e., total fungal biomass) were the result of complex interactions between fungal species number, fungal species identity (/identities), and bacterial presence (Fig. 1b; Appendix S2: Table S3). Biomass production capacity of the four model fungi, expressed as ergosterol concentrations, ranged from 47 to 261 μg/g leaf dry weight (Fig. 1b) and followed the same pattern as the fungi's degradative capacities (Fig. 1a; Appendix S2: Fig. S1). Growth rate ranged, on average, from 3.33 to 18.65 μg ergosterol/g leaf dry weight/d (Appendix S2: Table S1) and tended to be a critical determinant for the functional separation between the species, though not statistically significant (Fig. 2a). Ergosterol concentrations in bacteria-free fungal mixed cultures (90.8–197.0 μg/g leaf dry weight) did not exceed the performance of the most productive monoculture (i.e., *T. marchalianum*:

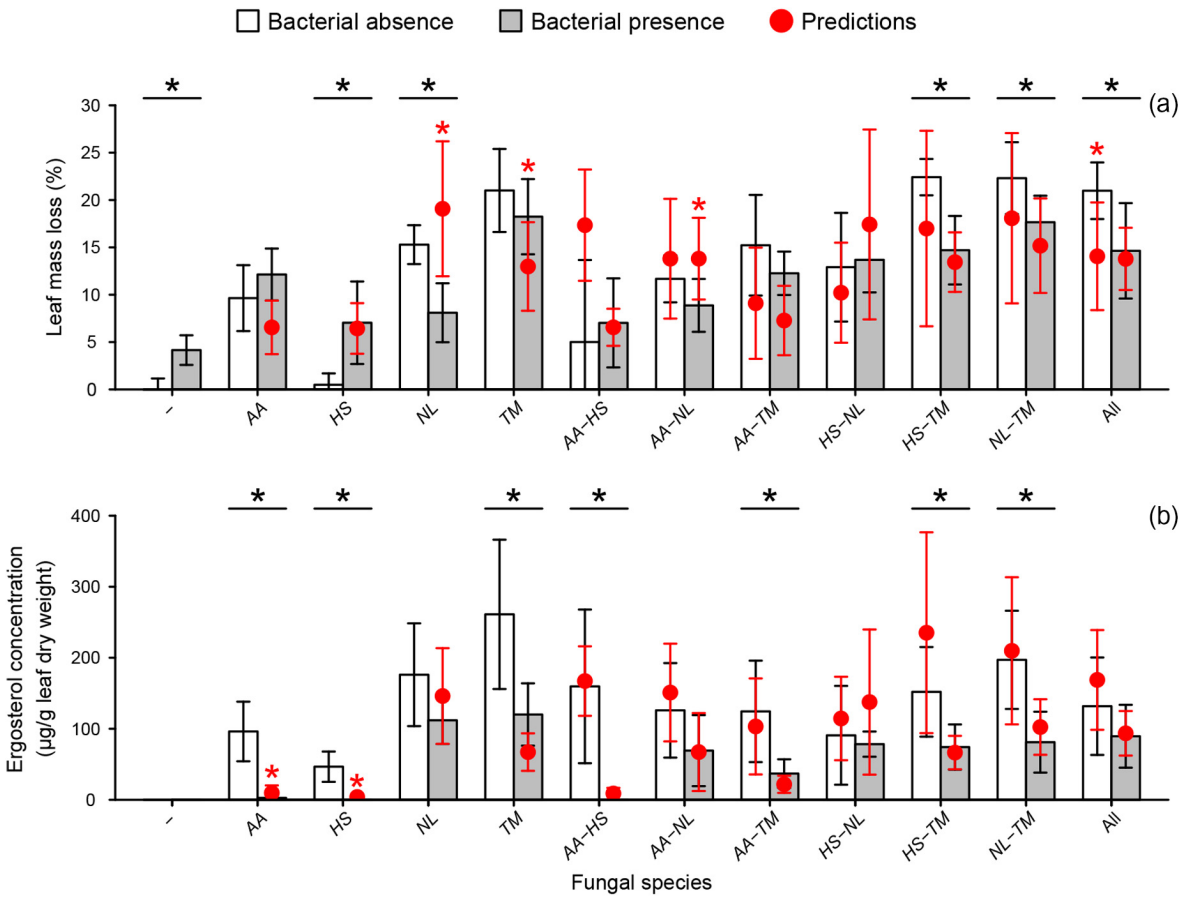

FIG. 1. (a) Mean leaf mass loss (in percent; relative to the initial leaf dry weight) and (b) ergosterol concentrations (in µg/g leaf dry weight; with 95% confidence intervals, respectively; $n = 6$) in fungal cultures in absence (white bars) and presence of bacteria (gray bars). Red points indicate predictions based on fungal and bacterial biomass–specific leaf mass loss or ergosterol concentrations. Red asterisks indicate significant differences between observed and predicted leaf mass loss or ergosterol concentrations of fungal treatments containing more than one organism (i.e., presence of bacteria or further fungi). Black asterisks indicate significant differences in leaf mass loss or ergosterol concentrations between bacteria-free and bacteria-containing cultures (*AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*).

261.1 µg/g leaf dry weight) and matched predictions (Fig. 1b). Ergosterol concentrations in bacteria-containing fungal cultures were constantly lower than in those being bacteria-free (Fig. 1b) and largely met predictions. The only exceptions were the bacteria-containing fungal monocultures *A. acuminata* and *H. stellata*, which showed significantly lower ergosterol concentrations (2.7 and 0.0 µg/g leaf dry weight, respectively) than predicted (9.61 and 3.28 µg/g leaf dry weight, respectively; Fig. 1b). Likely these deviations are not the result of ecological interactions. In fact, such low ergosterol concentrations—as predicted by amounts of DNA—could possibly be below the HPLCs' detection limit. In bacteria-only cultures, no ergosterol was detected (Fig. 1b), which indicates that the bacterial inoculum was not contaminated with true fungi (cf. Mille-Lindblom et al. [2006], Danger et al. [2013]).

Enzyme activities

The enzyme activities varied substantially depending on the treatment and changes in activities of all enzymes significantly contributed to the functional separation between cultures (Fig. 3; Appendix S2: Tables S4, S5). Mean activities ranged among the different cultures from 3.42 to 42.74 (PHO), 0.45 to 4.86 (AGL), 1.54 to 54.68 (BGL), 0.43 to 2.27 (CEL), 0.17 to 3.95 (XYL), 0.01 to 0.37 (PER), and 0.01 to 0.17 µmol/(h × g) leaf dry weight (PHE; detailed information on enzyme activities is provided in Appendix S2: Fig. S2). Note that absolute activities do not necessarily reflect the relative importance of an enzyme compared to others (e.g., BGL vs. PHE; Appendix S2: Fig. S2), because sample storage conditions and duration as well as differences in experimental and analysis temperature (16°C vs. 18°C) can affect activities in an enzyme-specific manner (German

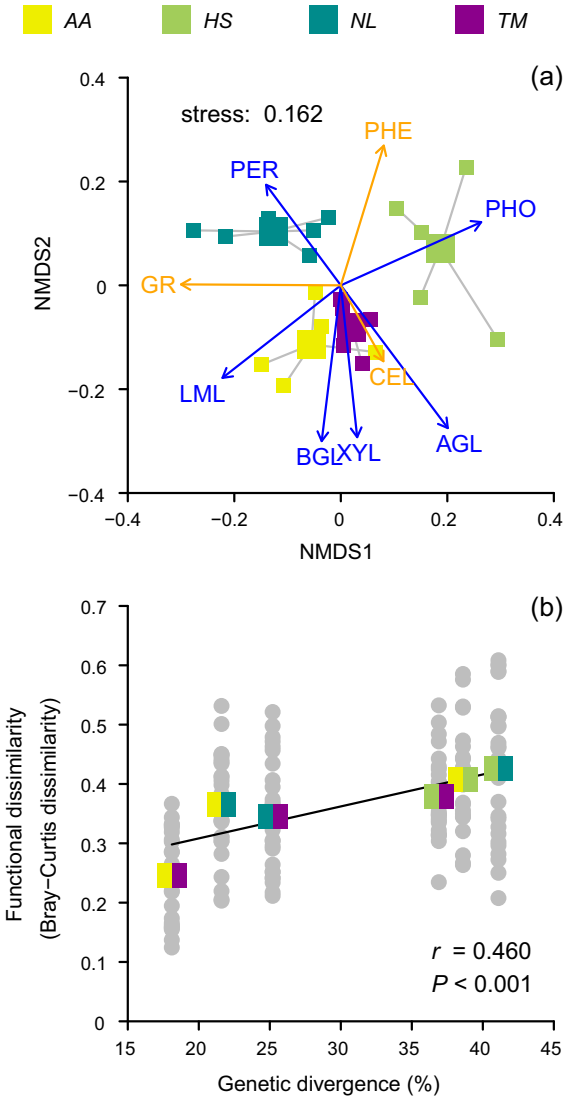

FIG. 2. (a) Non-metric multidimensional scaling (NMDS) plot for biomass-specific functional performances of the fungi (i.e., leaf mass loss [LML], enzyme activities [for abbreviations see below], and growth rates [GR] based on ergosterol concentrations) using Bray–Curtis dissimilarity. Group centroids and replicates are displayed as large and small squares, respectively. Blue and orange arrows display functional performances significantly and non-significantly contributing to the separation between the species, respectively (LML: $P = 0.002$; GR: $P = 0.096$; PHO: $P = 0.001$; AGL: $P = 0.001$, BGL: $P = 0.001$; CEL: $P = 0.521$; XYL: $P = 0.001$; PER: $P = 0.011$; PHE: $P = 0.122$). The provided stress value indicates a reasonable fit (i.e., <0.2; Clarke 1993). (b) Correlation between functional dissimilarity (Bray-Curtis dissimilarity) and genetic divergence of pairs of fungi (grey circles; dissimilarities were calculated for all possible replicate combinations of two cultures). Mean functional dissimilarities between two fungi are displayed as colored squares. Pearson's correlation coefficient (r) and P-value indicate a weak but statistically significant positive correlation (Hinkle et al. 2003). The regression line was added to visualize the relationship between the variables (*AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*; PHO, phosphatase; AGL, α-1,4-glucosidase; BGL, β-1,4-glucosidase; CEL, cellobiohydrolase; XYL, β-1,4-xylosidase; PER, peroxidase; PHE, phenol oxidase). Note the reduced sample size for *A. acuminata*, *H. stellata*, and *T. marchalianum* due to missing ergosterol or enzyme activity data for one replicate, respectively ($n = 5$).

et al. 2011). Significant impacts of independent variables on enzyme activities were identified as follows: fungal diversity impacted PHO, BGL, XYL, and PER activities; fungal species identity impacted AGL, BGL, CEL, XYL, PER, and PHE activities; bacterial presence impacted AGL, CEL, XYL, PER, and PHE activities; the interaction of fungal diversity and bacterial presence only impacted AGL activity; and the interaction of fungal species identity and bacterial presence impacted AGL, BGL, XYL, and PER activities (Appendix S2: Table S4). The fungi were capable of producing all of the investigated enzymes (Fig. 3; Appendix S2: Fig. S2), whereas the enzymatic capabilities of the fungi considered as ecologically meaningful functional trait (for definition see Statistical analyses) varied among species (Appendix S2: Table S1). Functional separation of the species was primarily driven by changes in activities of PHO, AGL, BGL, XYL, and PER (significant) and to a lesser extent by CEL and PHE (non-significant; Fig. 2a). Enzyme activities in bacteria-free fungal mixed cultures did not exceed performances of the most productive monocultures (i.e., *N. lugdunensis*; Appendix S2: Fig. S2f, g; and *T. marchalianum*; Appendix S2: Fig. S2a–e), and only deviated in approximately 10% of the comparisons from the predictions (Appendix S2: Fig. S2), indicating the prevalence of additive relationships. Also, the bacteria-only culture was capable of producing all of the investigated enzymes, whereas only AGL, BGL, CEL, and XYL were significantly different from the sterile control (Appendix S2: Fig. S2). The average enzyme activity in the bacteria-only culture was 2.4-fold lower compared to the average of the four fungal monocultures (Appendix S2: Fig. S2), which approximately corresponds to the observed difference in leaf decomposition performance between these organism groups (i.e., a 2.8-fold difference). Compared to bacteria-free cultures, bacterial presence induced a shift in enzyme profiles towards higher AGL, BGL, CEL, and XYL activities and lower PHO and oxidase activities (PER and PHE; Fig. 3). In approximately 20% of comparisons, enzyme activities in bacteria-containing fungal cultures significantly deviated from predictions, indicating a higher frequency of interactions in bacteria-containing fungal cultures than in bacteria-free fungal mixed cultures (Appendix S2: Fig. S2). The majority of these significant deviations was identified for PER and PHE activities (12 out of 17), which generally showed—along with PHO activity—the highest variability among enzymes (Appendix S2: Fig. S2a, f, g).

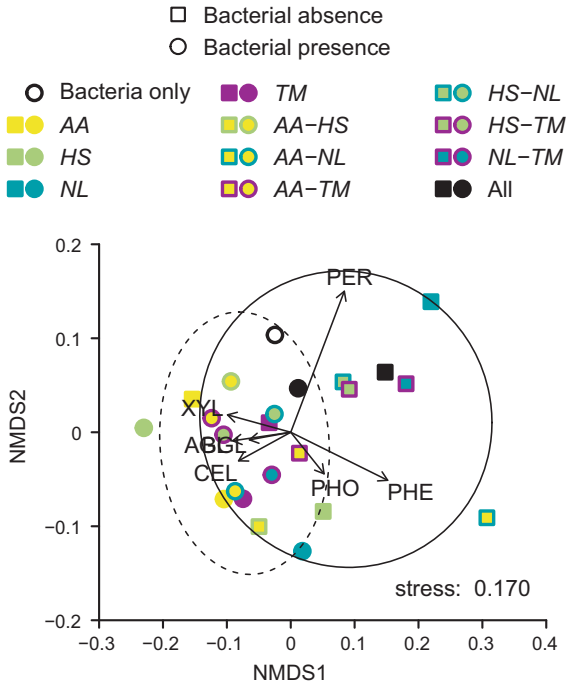

FIG. 3. Non-metric multidimensional scaling (NMDS) plot for enzyme activities using Bray–Curtis dissimilarity. Group centroids are displayed as colored symbols (for individual replicates see Appendix S2: Fig. S4). Squares and circles indicate bacterial absence and presence, respectively. Solid and dashed ellipses indicate group clusters of cultures in absence and presence of bacteria, respectively. Arrows display enzyme activities significantly contributing to the separation between treatments ($P = 0.001$–0.003). The provided stress value indicates a reasonable fit (i.e., <0.2; Clarke 1993; *AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*; PHO, phosphatase; AGL, α-1,4-glucosidase; BGL, β-1,4-glucosidase; CEL, cellobiohydrolase; XYL, β-1,4-xylosidase; PER, peroxidase; PHE, phenol oxidase).

Genetic divergence and functional dissimilarity

Genetic divergences based on base substitutions in the ITS region ranged from 18% to 41%. Functional dissimilarities based on Bray–Curtis dissimilarity indices ranged from 0.25 to 0.43 (Fig. 2b; Table 1). The correlation between genetic divergence and functional dissimilarity among the strains was weak but significantly positive (Fig. 2b).

Community composition and leaf colonization

Fungal diversity, species identity (of co-occurring species), and bacterial presence were significant determinants for DNA concentrations of all fungi (Fig. 4; Appendix S2: Table S6). Only for *H. stellata*, effects on DNA concentrations did result from an interaction of fungal diversity and bacterial presence (Fig. 4b; Appendix S2: Table S6). For all fungi but *T. marchalianum*, effects on DNA concentrations were affected by the interaction of species identity and bacterial presence (Fig. 4d; Appendix S2: Table S6). Irrespective of bacterial absence or presence, DNA concentrations of each fungus were in mixed cultures lower than in monoculture, except for *A. acuminata* in the bacteria-free binary culture *A. acuminata–H. stellata* (Fig. 4a). Compared to monocultures, DNA concentrations of *A. acuminata*, *H. stellata*, *N. lugdunensis*, and *T. marchalianum* in fungal mixed cultures were, in the absence of bacteria, on average 56%, 90%, 30%, and 69% lower, respectively, and in the presence of bacteria on average 47%, 58%, 52%, and 23% lower, respectively (Fig. 4). Generally, compared to bacteria-free cultures, the presence of bacteria resulted in reduced DNA concentrations in fungal monocultures and mixed cultures with average reductions of 44%, 73%, 36%, and 53% for *A. acuminata*, *H. stellata*, *N. lugdunensis*, and *T. marchalianum*, respectively (Fig. 4).

Fungal leaf colonization, expressed as NDEs on DNA concentrations, was, in bacteria-free fungal mixed cultures, on average negative (−13.5%) and ranged from −56.7% to 114.6% among the cultures (Fig. 5). NDEs were mainly composed of trait-independent complementarity effects (explaining 113.8% of NDEs), whereas dominance and trait-dependent complementarity effects were opposed to NDEs (explaining −7.6% and −6.2% of NDEs, respectively; Fig. 5). NDEs significantly correlated with the average growth rate and genetic divergence of the mixed cultures (negatively and positively, respectively; Fig. 6a,

TABLE 1. Genetic divergence (GD) and functional dissimilarities (FD) between the fungal species.

	Alatospora acuminata		*Heliscella stellata*		*Neonectria lugdunensis*		*Tetracladium marchalianum*	
Species	GD	FD	GD	FD	GD	FD	GD	FD
A. acuminata	0	0.00						
H. stellata	39	0.41	0	0.00				
N. lugdunensis	22	0.36	41	0.43	0	0.00		
T. marchalianum	18	0.25	37	0.38	25	0.35	0	0.00

Notes: Estimates of genetic divergence between the fungi are based on ITS sequences. The percentage of base substitutions between sequences is shown. Analyses were conducted using the maximum composite likelihood model. Mean functional dissimilarities based on biomass-specific functional performances were calculated using Bray–Curtis dissimilarity.

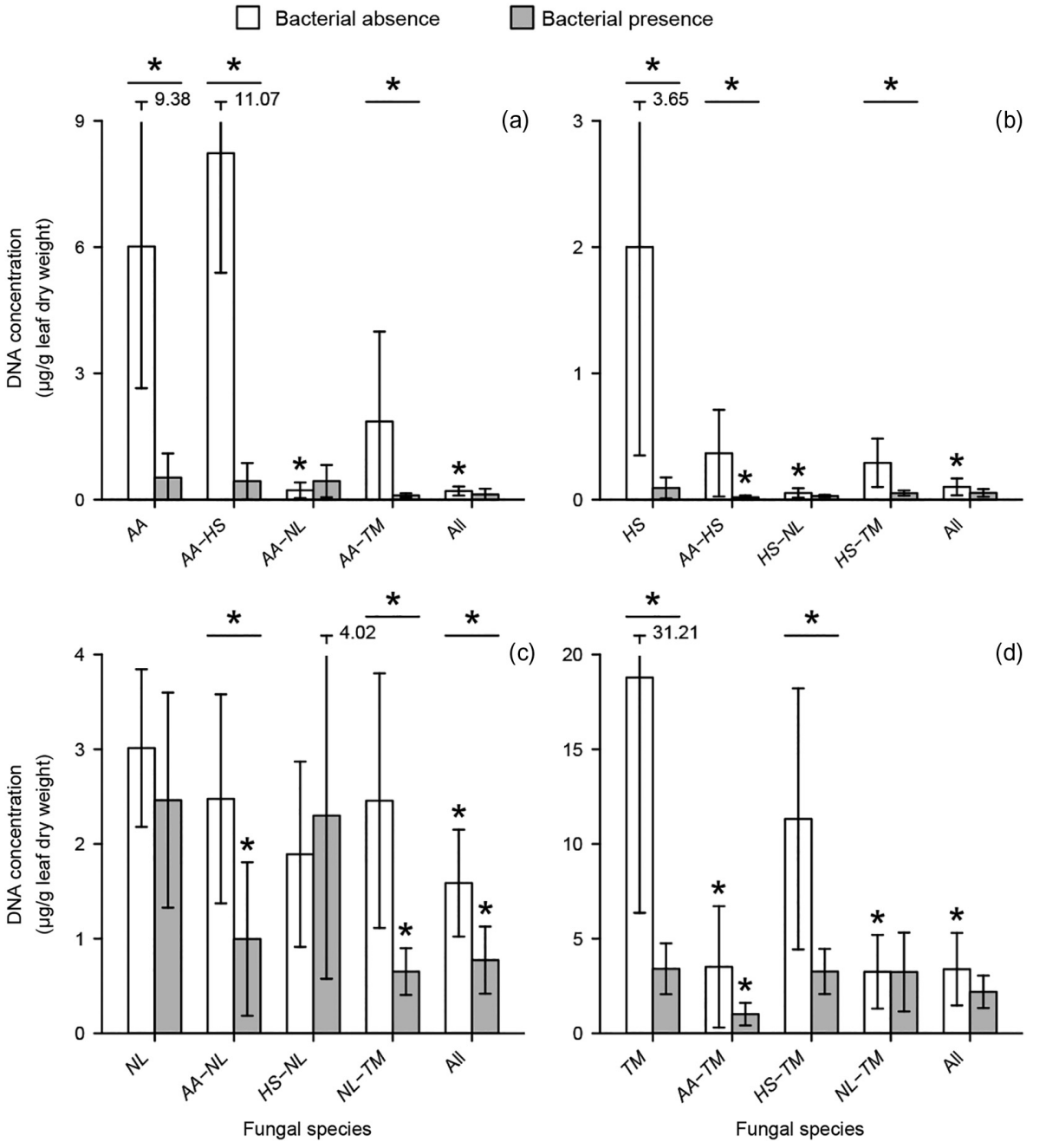

FIG. 4. Mean species-specific DNA concentrations (µg DNA/g leaf dry weight) of (a) *A. acuminata*, (b) *H. stellata*, (c) *N. lugdunensis*, and (d) *T. marchalianum* (with 95% confidence intervals) in mono, binary, and quaternary cultures in the absence (white bars) and presence of bacteria (gray bars). Asterisks above bars indicate significant differences to the monoculture within the same bacteria treatment and asterisks above horizontal lines indicate significant differences between bacteria-free and bacteria-containing cultures within the same fungal combination (*AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*).

b). Pairwise genetic distances between the fungi ranged from 18% to 41% (Table 1) and the overall mean distance was 30%. With one exception (i.e., the binary culture *A. acuminata–H. stellata*), NDEs were (partially) significantly higher in bacteria-containing than in bacteria-free fungal mixed cultures and were on average positive (42.8%), ranging between −43.4% and 93.8% among cultures (Fig. 5). These NDEs were, in the presence of bacteria, mainly composed of trait-independent complementarity and dominance effects (explaining

50.6% and 33.7% of NDEs, respectively; Fig. 5), prevailing over trait-dependent complementarity effects (explaining 15.6% of NDEs; Fig. 5). In the presence of bacteria, NDEs were not correlated with cultures' average growth rate, but still showed a slight positive dependence on genetic divergence (Fig. 6c, d).

Finally, bacterial densities ranged from 66×10^9 to 113×10^9 cells per mg leaf dry weight among cultures that received bacterial inoculum (Appendix S2: Fig. S3). Fungal diversity and species identity had no significant impact on bacterial cell densities (Appendix S2: Table S7).

Discussion

Functional characterization of the fungi and the bacterial community

Although both fungi and bacteria were capable of decomposing leaf material and producing the investigated enzymes, fungal cultures were on average more productive (Fig. 1a; Appendix S2: Fig. S2). Our results indicate that both organism groups exploit similar resources (Fig. 3; Appendix S2: Fig. S2), potentially causing antagonistic (e.g., competitive) interactions, which are predominantly reported on submerged plant material (Gulis and Suberkropp 2003, Mille-Lindblom and Tranvik 2003, Mille-Lindblom et al. 2006, Romaní et al. 2006).

As observed earlier, fungi show substantial differences in leaf mass loss (Fig. 1a; e.g., Andrade et al. [2016], Bärlocher and Corkum [2003]), which are largely reflected by fungal biomass production (Fig. 1b; Appendix S2: Fig. S1). This suggests the species' growth rate as a major trait determining their OFP (Gessner et al. 2010, Aguilar-Trigueros et al. 2015). Furthermore, marked differences in enzyme activities among fungi (Fig. 2a; Appendix S2: Table S1) suggest different functional roles supporting synergistic (e.g., complementary) interactions. Although the leaf decomposition efficiency was nearly identical for *A. acuminata*, *N. lugdunensis*, and *T. marchalianum*, the different patterns in enzyme activities point to a qualitative difference among species and the occupation of different ecological niches (Fig. 2a). *Alatospora acuminata* and *T. marchalianum*, however, exhibit a very similar pattern in enzyme activities dominated by hydrolytic enzymes degrading phosphate esters, oligosaccharides, cellulose, and hemicellulose (Fig. 2a; Appendix S2: Fig. S2, Table S1). Consequently, both species are assumed to exploit similar resources (i.e., niche similarity), which is also reflected by their low genetic divergence and functional dissimilarity (Fig. 2, Table 1; Andrade et al. 2016) as well as their placement in the same taxonomic class (i.e., Leotiomycetes [Baschien et al. 2013]).

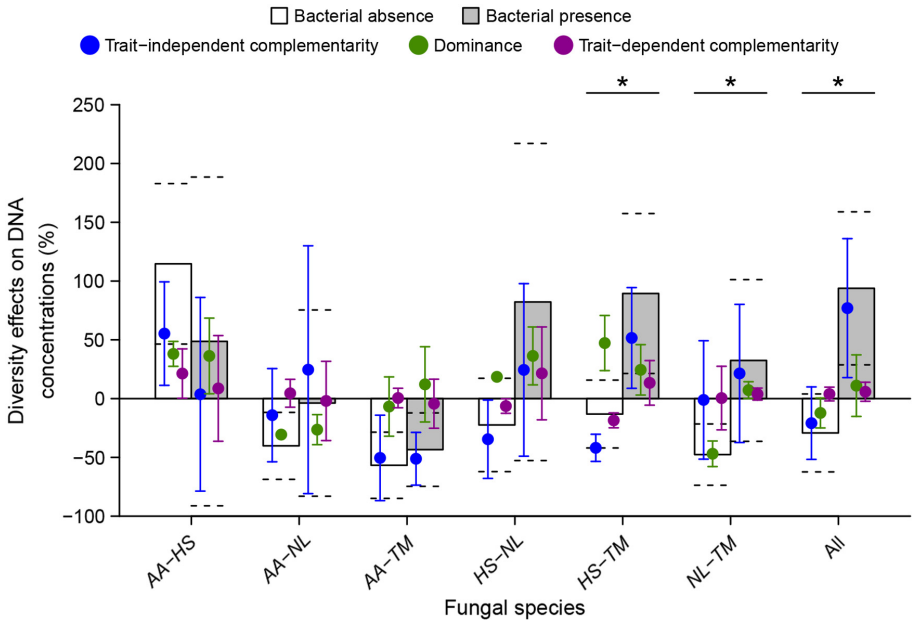

Fig. 5. Mean diversity effects on DNA concentrations (in percent; with 95% confidence intervals; $n = 6$) in fungal mixed cultures based on a modified Price equation (i.e., tripartite partition; Fox [2005]). Bars indicate mean net diversity effects on fungal leaf colonization in absence (white bars) and presence of bacteria (gray bars), which are composed of the sum of trait-independent complementarity (blue points), dominance (green points), and trait-dependent complementarity effects (purple points). Asterisks indicate significant differences in net diversity effects between bacteria-free and bacteria-containing cultures (*AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*).

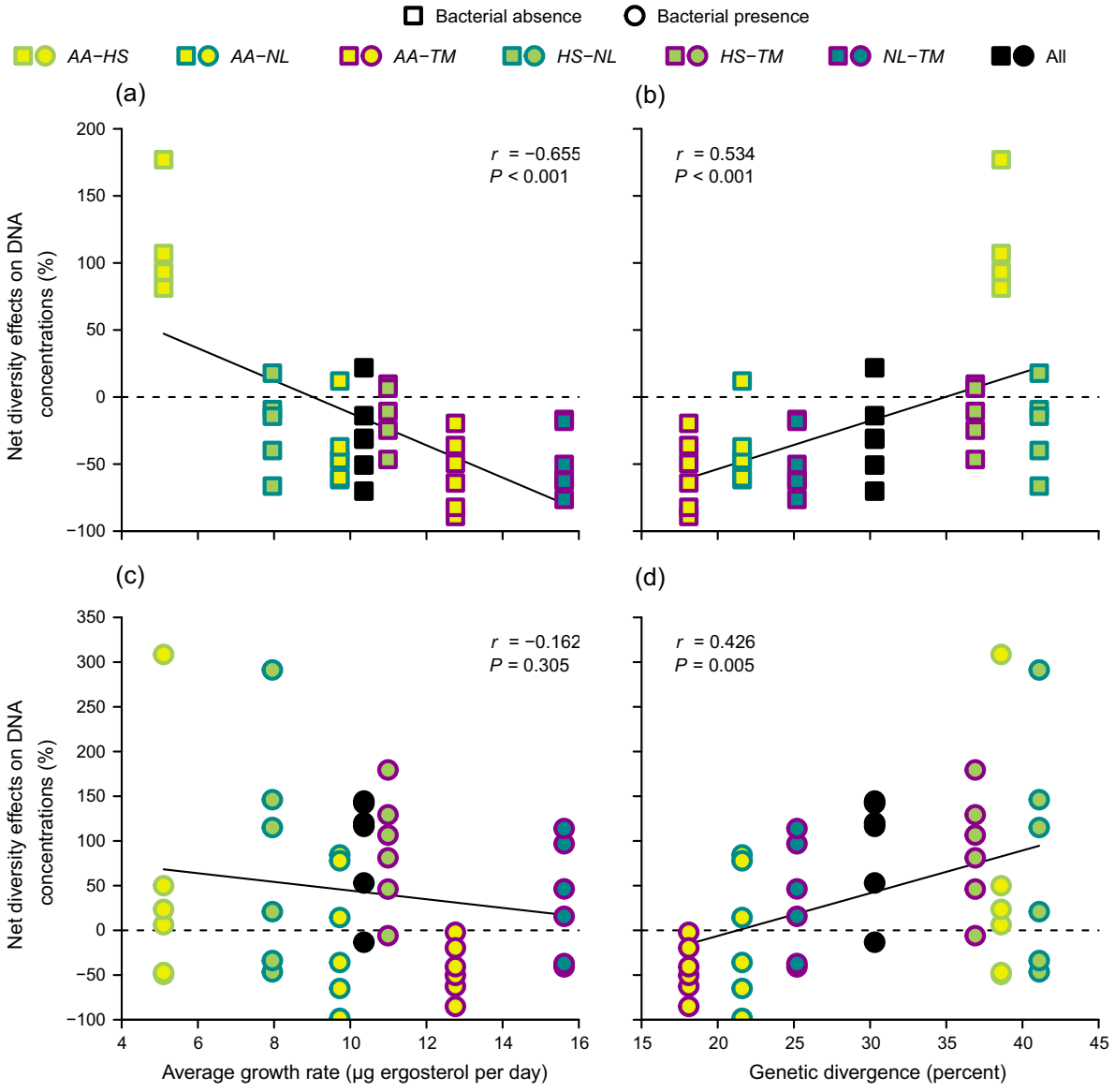

FIG. 6. Correlations between net diversity effects on DNA concentrations and average growth rates (a), (c) or genetic divergence (b), (d) of fungal mixed cultures in bacterial absence (a), (b) and presence (c), (d) with Pearson's correlation coefficients (*r*) and *P* values. The regression lines were added to visualize the relationships between the variables (*AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*).

Leaf decomposition by *N. lugdunensis*, in contrast, is based on a low and high activity of hydrolytic and oxidative enzymes, respectively (Fig. 2a; Appendix S2: Fig. S2, Table S1). The latter degrade lignin, the most recalcitrant plant polymer (Kaltschmitt et al. 2016), accounting for up to 18% of dry mass in black alder leaves (Lecerf and Chauvet 2008). However, contrary to our findings, oxidative enzymes are assumed to play a minor role in enzyme inventories of aquatic fungi colonizing leaves (Gessner et al. 2010). The functional traits of these three species indicate niche differentiation and thus synergistic interactions among *N. lugdunensis* and *A. acuminata* or rather *T. marchalianum* (Fig. 2). Relative to the other three fungal species, the leaf decomposition efficiency of *H. stellata* was 10 times lower (Appendix S2: Table S1). This observation suggests that *H. stellata* primarily utilizes other energy resources, such as dissolved organic compounds, for growth instead of solid substrate (Fig. 1a). Moreover, *H. stellata* shows only pronounced activity of those enzymes that are involved in the degradation of less recalcitrant low-molecular-weight organic compounds (Fig. 2a; Appendix S2: Fig. S2, Table S1), such as phosphate esters or maltose, which are readily leached from leaves or released during advancing leaf senescence (Wildman and Parkinson 1981, Baldwin 1999, Duan et al. 2014). This functional differentiation of *H. stellata* from

the other fungi (Appendix S2: Table S1) suggests niche differentiation, which is supported by a high genetic divergence and functional dissimilarity (Fig. 2b; Table 1).

All in all, these data highlight substantial differences in the enzymatic capacities among the fungal species, despite this being generally considered rare in aquatic fungi (Gessner et al. 2010). These findings are highly relevant for B-EF research but require further attention to understand transferability to other environmental conditions and a broader range of species.

Characterization of fungal–fungal interactions

Fungal mixed cultures did not (or barely) surpass OFPs in terms of leaf mass loss, fungal biomass production, and enzyme activities of the most productive monocultures (i.e., *N. lugdunensis* and *T. marchalianum*; Fig. 1; Appendix S2: Fig. S2). This observation is in line with earlier B-EF studies on aquatic fungi (e.g., Bärlocher and Corkum [2003], Duarte et al. [2006], Andrade et al. [2016]). However, earlier studies were not able to quantify individual species' biomasses, making it difficult to unravel the underlying processes (complementarity vs. dominance or additivity vs. interactions). As a consequence of methodological developments (Baudy et al. 2019), this study demonstrates that interactions (i.e., NDEs) occur during leaf colonization and highly depend on species identity, whereas the OFP of communities is defined by the sum of individual species' performance (i.e., additivity) and thus by community composition.

Fungal leaf colonization, expressed as NDEs on DNA concentrations, was in mixed cultures largely negative and driven by negative trait-independent complementarity effects (Fig. 5), indicating competition for similar resources (niche overlap) at mutual expense (Fox 2005). This result is in contrast to the synergistic interactions predicted from fungal traits (see Functional characterization of the fungi and the bacterial community; Fig. 2; Appendix S2: Table S1). Nonetheless, differences in NDEs on leaf colonization among the mixed cultures may be explained by the average fungal growth rate and genetic divergence of the communities, which are significantly negatively and positively correlated with these NDEs, respectively (Fig. 6a,b). The negative relationship between NDEs on leaf colonization and fungal growth rate may result from the exclusion of environmental constraints, as for instance imposed by competition with other microorganism groups or consumption by invertebrates (Bärlocher 1980, Mille-Lindblom and Tranvik 2003), allowing rapid fungal colonization of the leaf substrate. Accordingly, species with higher growth rates may rapidly colonize leaves up to their carrying capacity, thereby preventing or limiting colonization of species with lower growth rates (Fig. 4). Such interactions—referred to as inhibitory priority effects (Fukami 2015)—may, however, be the result of the experimental conditions (i.e., exclusion of environmental constraints), possibly masking facilitative interactions occurring under field conditions (cf. Gessner et al. 2010). The positive relationship between NDEs on leaf colonization and genetic divergence supports the findings of Andrade et al. (2016), suggesting a close link between genetic and functional diversity in aquatic fungal communities (for terrestrial communities, see also Eichlerová et al. [2015]). Accordingly, and although these NDEs were mainly negative (Fig. 5), antagonistic interactions were less pronounced between genetically and functionally distinct species (see previous subsection), being thus more likely to coexist on leaves.

OFPs in terms of leaf decomposition, fungal biomass production, and enzyme activities in mixed cultures could largely be predicted by individual species' B-SFPs (Fig. 1; Appendix S2: Fig. S2), indicating that the functioning of these communities is based on additivity. However, there are exceptions. In the binary culture *A. acuminata*–*H. stellata*, *A. acuminata* was the only species in this experiment, showing a higher biomass in mixed cultures than in monoculture (Fig. 4a). Yet, leaf decomposition was (close to significant) more than two times lower than predicted (Fig. 1a), and enzyme activities tended to be lower as well (Appendix S2: Fig. S2). This indicates a more efficient conversion of leaf substrate (but also dissolved organic compounds, as presumed for *H. stellata*) to fungal biomass by the joint action of these species, which might be attributed to a complementary resource use supported by their distinct enzyme inventories. In contrast, in the quaternary species combination, leaf decomposition was significantly higher than predicted (Fig. 1a), whereas the observed enzyme activities largely met the predictions (Appendix S2: Fig. S2). This points to a more efficient decomposition of leaf substrate by the joint activity of a diverse enzymatic pool provided by the four species, which can also be interpreted as complementary interaction (cf. Pascoal et al. [2010]). As indicated by the relatively low ergosterol concentration (cf. *T. marchalianum* in monoculture; Fig. 1b), however, the excess of decomposition products was seemingly not converted into fungal biomass, but may possibly have been utilized to meet increased energy demands for physiological responses involved in competitive interactions among fungi (Chan et al. 2019). Such energetically costly responses include, for instance, an enhanced nutrient uptake and metabolism or protein stabilization and recycling (Ujor et al. 2018).

Characterization of fungal–bacterial interactions

As hypothesized, compared to bacteria-free cultures, bacterial presence largely resulted in lower leaf decomposition and an inhibition of fungal growth (Fig. 1; Gulis and Suberkropp 2003), which may be the result of competition (Mille-Lindblom et al. 2006). The magnitude of this effect, however, highly depends on the number and identity of fungal species (Appendix S2: Table S3). Compared to bacteria-free cultures, oxidase activities were lower in

the presence of bacteria (Fig. 3); although against our hypothesis, hydrolase activities were by tendency increased (Fig. 3; Appendix S2: Fig. S2). Thus, the lower leaf decomposition observed in the presence of bacteria may be based on lower oxidase activities. This indicates that ligninolytic capacities could play a greater role in leaf decomposition by aquatic fungi than previously assumed (cf. Gessner et al. [2010], Bärlocher and Boddy [2016]). These lower (overpredicted) oxidase activities in presence of bacteria (Appendix S2: Fig. S2) may possibly be explained by a release of bacterial metabolites functioning as oxidase inhibitors (Fernandes and Kerkar 2017). As bacterial presence affected biomass production of rapidly growing fungi to a lesser extent (i.e., *N. lugdunensis* and *T. marchalianum*; Fig. 1b), the growth rate appears to be a crucial trait determining the resistance of aquatic fungi in competition with bacteria (but see Mille-Lindblom and Tranvik [2003], Mille-Lindblom et al. [2006]). Accordingly, priority effects may also play an important role in the interplay between bacteria and fungi during leaf colonization.

Although bacterial presence generally seemed to inhibit fungal OFPs, NDEs on leaf colonization in fungal mixed cultures shifted from negative—as observed in bacteria-free mixed cultures—to positive (Fig. 5). These positive NDEs are mainly driven by positive trait-independent complementarity effects (Fig. 5), suggesting the prevalence of synergistic interactions between fungi (Fox 2005), which may have been induced by the presence of bacteria. Bacterial inhibition of fungal growth may alleviate inhibitory priority effects among fungi during colonization. This enables fungal species with lower growth rates (i.e., *A. acuminata* and *H. stellata*) to colonize the substrate under lower competitive pressure exerted by species with higher growth rates (i.e., *N. lugdunensis* and *T. marchalianum*; Figs. 4 and 5). Accordingly, bacteria may have induced new fungal diversity-based feedback loops that promote coexistence and synergistic interactions as predicted from fungal traits (see Functional characterization of the fungi and the bacterial community). In support of this statement, fungal growth rates, which explained interactions in bacteria-free cultures, were in presence of bacteria no longer explanatory for differences in NDEs on leaf colonization (Fig. 6c). In contrast, the differences in these NDEs were in presence of bacteria still positively correlated with genetic divergence in fungal mixed cultures (Fig. 6d), which provides further evidence for the hypothesized link between genetic and functional diversity in aquatic fungal communities (Fig. 2; Andrade et al. 2016), also under more environmentally realistic conditions.

In the presence of bacteria, OFPs in fungal monocultures and mixed cultures were again largely determined by the sum of contributing organisms' B-SFPs (i.e., by community composition; Fig. 1; Appendix S2: Fig. S2). However, in a few instances, observations clearly deviated from predictions, mainly concerning leaf decomposition in culture combinations comprising fungal monocultures and bacteria (Fig. 1a). In the presence of bacteria, leaf decomposition in the *A. acuminata* and *T. marchalianum* cultures was substantially higher than predicted, which again points to an increased allocation of energy to physiological processes involved in competitive interactions by fungi and bacteria. In contrast, co-occurrence of bacteria and *N. lugdunensis*—the model fungus most resistant to bacteria (Fig. 1b)—resulted in a more than two times lower leaf decomposition than predicted (Fig. 1a). This indicates synergistic interactions between the fungus and bacteria, which may be expressed by a higher efficiency in the assimilation of leaf substrate by *N. lugdunensis*.

Conclusion

The application of novel qPCR assays uncovered that fungal–fungal interactions are determined by functional and genetic divergence. Specifically, niche complementarity does not necessarily lead to positive biodiversity effects on substrate colonization or functioning within fungal communities. The presence of bacteria likely resulted in new fungal diversity-based feedback loops. These eventually promote fungal coexistence and complementary functioning, irrespective of the apparent antagonistic relationship between bacteria and fungi. Although fungi are considered as suitable model organisms to address key questions in community ecology (Zubrod et al. 2019), our study highlights the necessity to conduct future B-EF microcosm studies on fungi not under exclusion of naturally co-occurring key organism groups within the microbial compartment of interest. Otherwise, study outcomes might likely not reflect true ecological relationships as they occur in the environment and ultimately lead to misguided strategies for the conservation of biodiversity and ecosystem processes.

Although in this experiment the culture with the highest diversity (i.e., quaternary fungal species combination in presence of bacteria) was outperformed by a range of cultures with a lower diversity, species-rich communities are assumed to bear a higher capacity to buffer effects of environmental change on microbial functioning than species-poor communities do (Fernandes et al. 2011, Gonçalves et al. 2015). In the light of climate change and biodiversity loss, however, this buffering capacity might be substantially reduced, which poses a risk to the integrity of aquatic ecosystems. To be able to conserve these systems, a mechanistic understanding of relationships between biodiversity, resilience, and ecosystem functions in microbial decomposer communities facing anthropogenic stress is essential and should be pursued in future studies.

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