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Elevated Fungicide and Nutrient Concentrations Change Structure but not Function of Aquatic Microbial Communities

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Abstract: Leaf decomposition is a key process in stream ecosystems within forested catchments; it is driven by microbial communities, particularly fungi and bacteria. These microorganisms make nutrients and energy bound in leaves available for wider parts of the food web. Leaf-associated microorganisms are subjected to anthropogenic pressures, such as the increased exposure to nutrients and fungicides associated with land-use change. We assessed the sensitivity of leaf-associated microbial communities with differing exposure histories, namely, from pristine (P) streams, and streams impacted by wastewater (W) and agricultural run-off (vineyards; V). In the laboratory, microbial communities were exposed to elevated nutrient (NO_3-N: 0.2–18.0 mg/L, PO_4-P: 0.02–1.8 mg/L) and fungicide concentrations (sum concentration 0–300 µg/L) in a fully crossed 3 × 4 × 4-factorial design over 21 days. Leaf decomposition and exoenzyme activity were measured as functional endpoints, and fungal community composition and microbial abundance served as structural variables. Overall, leaf decomposition did not differ between fungicide treatments or exposure histories. Nonetheless, substantial changes in the fungal community composition were observed after exposure to environmentally relevant fungicide concentrations. Elevated nutrient concentrations assisted leaf decomposition, and the effect size depended on the exposure history. The observed changes in the fungal community composition support the principle of functional redundancy, with highly efficient decomposers maintaining leaf decomposition. *Environ Toxicol Chem* 2024;43:1300–1311. © 2024 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Community structure; Exposure history; Land use; Leaf decomposition

INTRODUCTION

Leaf litter of terrestrial origin represents a significant energy source for aquatic ecosystems, such as rivers and streams within forested catchments (Fisher & Likens, 1973). The energy stored in leaf litter is made available to wider parts of the food web through leaf decomposition, which represents a key ecosystem process (Minshall, 1967; Nelson & Scott, 1962). For this process, bacteria and fungi are considered central (Dighton & White, 1983; Webster, 2007). Through their extracellular enzymatic capability, these microorganisms convert recalcitrant oligo- and polysaccharides into assimilable mono- and disaccharides, ultimately fuelling a wider part of the food web (Boulton & Boon, 1991; Hieber & Gessner, 2002).

Leaf decomposition in rivers and streams is, however, influenced by the catchments' land use and associated stressors. For example, the influx of nutrients and pesticides into surface waters, which have been linked to agricultural land use (Tilman et al., 2001), affects leaf-associated microbial communities. Whereas nutrients generally stimulate microbial activity up to a certain concentration (Ferreira et al., 2015), fungicides are mainly associated with a reduction in leaf decomposition (see Fernández et al., 2015; Zubrod, Feckler, et al., 2015; Zubrod, Schäfer, et al., 2015). Moreover, the microbial communities' functional response to fungicides and nutrients is influenced by the communities'

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exposure histories (Feckler et al., 2018; Gardeström et al., 2016). For example, the functional tolerance of leaf-associated microbial communities toward fungicides, measured through their leaf decomposition rate, was observed to be higher when sampled from streams impacted by agriculture (i.e., with exposure history) compared with near-natural streams (i.e., without exposure history; Feckler et al., 2018). This observation suggests that previous exposure to fungicides acts as a filter selecting for tolerant (and partly more efficient in terms of leaf decomposition) species, with the fungal group of aquatic hyphomycetes (AH) being considered as major driver of leaf decomposition (Gessner et al., 2007).

An earlier study (Feckler et al., 2018) acknowledged that the general applicability of their findings might be limited. Indeed, Feckler et al. (2018) employed only one site and thus one community for each of the two exposure histories, the latter being determined by the dominating land use surrounding that site. Our study expands the data set by sampling from streams associated with different land uses, providing a more robust basis of comparison for earlier findings. Leaf-associated microbial communities were sampled from pristine (P) streams, and streams impacted by wastewater (W) or run-off from the locally dominating crop, namely, vineyards (V), each independently replicated three times (i.e., nine sites in total). It was expected that leaf-associated microbial communities from V-impacted stream sections would be structurally and functionally adapted to moderate nutrient and high fungicide exposure, representing the major chemical stressors used in such catchments (Fernández et al., 2015; Tilman et al., 2001; Zubrod et al., 2019). Microbial communities impacted by W are expected to be adapted to relatively high nutrient concentrations while being exposed to a broad range of organic micropollutants including fungicides. Within the same sampling region, leaf-associated microbial communities sampled from P-streams were included to establish a baseline for the microbial communities' responses to fungicides and nutrients (Fernández et al., 2015).

In the laboratory, these microbial communities were exposed to environmentally relevant but elevated nutrient and fungicide concentrations, involving a fully crossed $3 \times 4 \times 4$-factorial design over 21 days. Besides microbially mediated leaf decomposition, we analyzed the communities' exoenzyme activities as well as fungal and bacterial abundances approximated by real-time quantitative polymerase chain reaction (PCR), and fungal community compositions through next-generation sequencing (NGS). We hypothesized (1) that microbially mediated leaf decomposition would be reduced with elevated fungicide levels, whereas the effects would be more pronounced for microbial communities from P-streams than for W- and V-streams (see Feckler et al., 2018); (2) that this leaf decomposition pattern would be reflected in a higher activity of enzymes degrading the recalcitrant carbon in W- and V- compared with P-communities, due to the colonization of leaves by more tolerant microbial communities with higher enzymatic capability (see Baudy et al., 2021); (3) that elevated nutrient levels would buffer the negative fungicide effects through the provisioning of additional and easily assimilable energy compared with treatments with lower nutrients (see Ferreira et al., 2015; but see Fernández et al., 2016); and (4) that finally, changes in leaf decomposition in response to elevated nutrient and fungicide exposure would be linked to shifts in the community structure (bacterial and fungal abundances and fungal community composition) favoring more tolerant and more efficient AH species; in this context, community changes were expected to be more prominent in P- than for W- and V-communities, with the latter two being already shaped through stressor exposures.

MATERIALS AND METHODS

General experimental design

The exposure histories of the leaf-associated microbial communities were defined by the land uses upstream of the sampling sites (Figure 1). Being aware that factors such as different soil properties, light availability, and photosynthetic differences at the sampling sites might change the properties of the leaf-associated microbial communities, they were considered as part of the factor "exposure history." The communities were sampled from pristine streams with forest-dominated catchments (P; sites P1, P2, and P3 as replicates), from streams mainly impacted by either wastewater discharge (W; sites W1, W2, and W3), or streams impacted by vineyard run-off (V; sites V1 and V2; severe draughts during autumn 2019 did not allow us to assess V3; see the Supporting Information, Table S1). We performed three independent semistatic bioassays, the first in April/May (sites P1, W1, and V1), the second in July/August (sites P2, W2, and V2) and the third in September/October (sites P3 and W3) 2019. Each of the bioassays was planned to include one community/exposure type (i.e., either P-, W-, or V-community), following a $3 \times 4 \times 4$-factorial design with a duration of 21 days (Figures 1–3 and Supporting Information, Table S1). Such a sequential procedure was employed because the number of experimental units for the entire experiment (i.e., 720) would not have been manageable in parallel.

Before the initiation of each of the bioassays in the laboratory, black alder (*Alnus glutinosa* (L.) Gaertn.) leaves collected for these experiments in the preceding years were deployed at the sampling sites to be colonized by the local community of microorganisms for 14 days (see the *Preparation of microbial inocula and leaf material* section; Figure 2). In the laboratory, these leaves were then homogenized to prepare a microbial inoculum for the exposure phase (Figure 2). Microorganisms were exposed to four concentrations of a fungicide mixture (0–300 µg/L; Supporting Information, Table S2; see the *Chemicals* section) crossed with four nutrient concentrations (Figure 3). The nutrient and fungicide mixture concentrations were selected based on previous studies, allowing for a direct comparison of results (Feckler et al., 2018; Zubrod, Feckler, et al., 2015). The nutrient medium composition largely followed Dang et al. (2005), but was adjusted in terms of NO_3-N (0.2, 2.0, 10.0, and 18.0 mg/L) and PO_4-P (0.02, 0.2, 1.0, and 1.8 mg/L) concentrations at a fixed ratio of 10 to 1 (Figure 3; Feckler et al., 2018). In the following text, these nutrient concentrations are referred to as very low, low, moderate, and high. The fully crossed design resulted in a total of 48 treatments, each replicated five times.

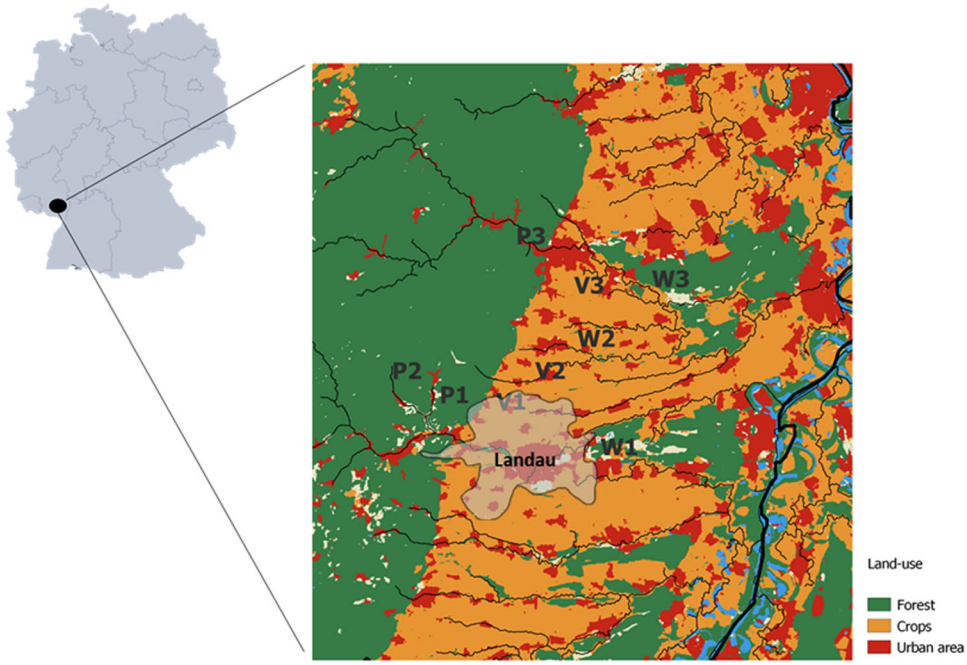

FIGURE 1: Map of the major land use for the sampling region near Landau, Germany. Green, orange, and red represent forest, crops, and urban area, respectively. Dark lines represent major stream segments. Letters represent different land-use categories upstream of the sampling sites, that is, pristine, P (1–3); wastewater treatment effluent, W (1–3); and vineyard, V (1–3) and their catchments based on Sentinel-2 10-m land-use map (Karra et al., 2021).

Preparation of microbial inocula and leaf material

The microbial inocula were obtained from streams near Landau, Germany (Supporting Information, Table S1 and Figure 1), by submerging thawed black alder leaves in litterbags (10 leaves with different sizes/bag; 15 × 15 cm; mesh size = 1 mm; $n = 50$) at each sampling site for 14 days (Figure 2). Leaf material originated from trees within the same region (49.20116°N, 8.09331°E) sampled shortly before abscission during autumn 2017 and 2018 and was visually inspected for

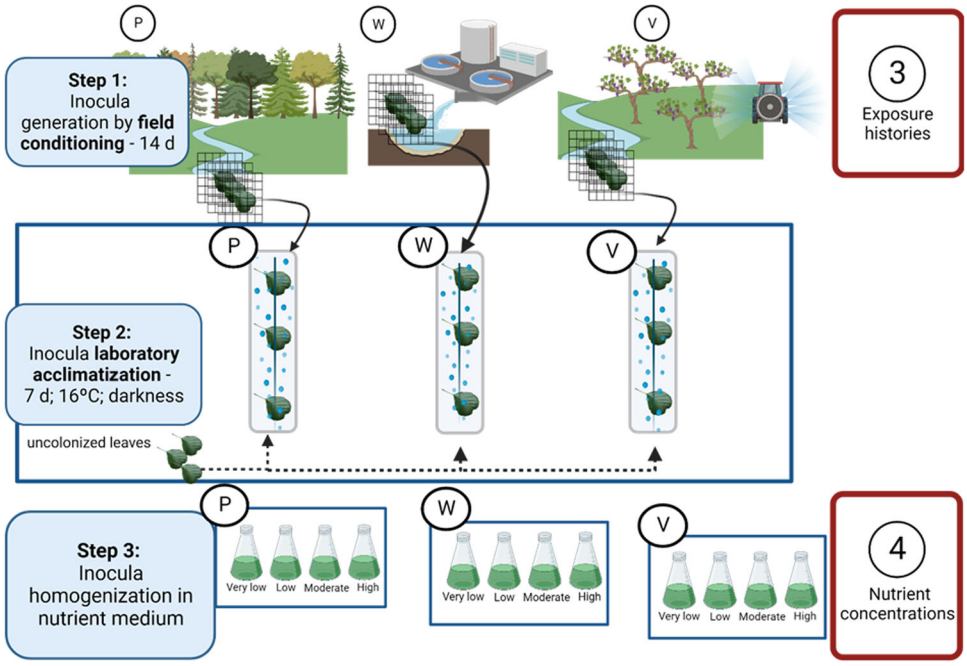

FIGURE 2: Schematic overview of the inocula preparation. Step 1: Generating inocula from pristine (P) streams, or streams impacted by wastewater discharge (W) and vineyard run-off (V) by deploying alder leaves in the field for 14 days; Step 2: Inocula acclimatization to laboratory conditions; leaves from each sampling site and uncolonized leaves are further microbially colonized for 7 days; Step 3: Inocula (leaves) homogenization in nutrient media/exposure history. Created with BioRender.com.

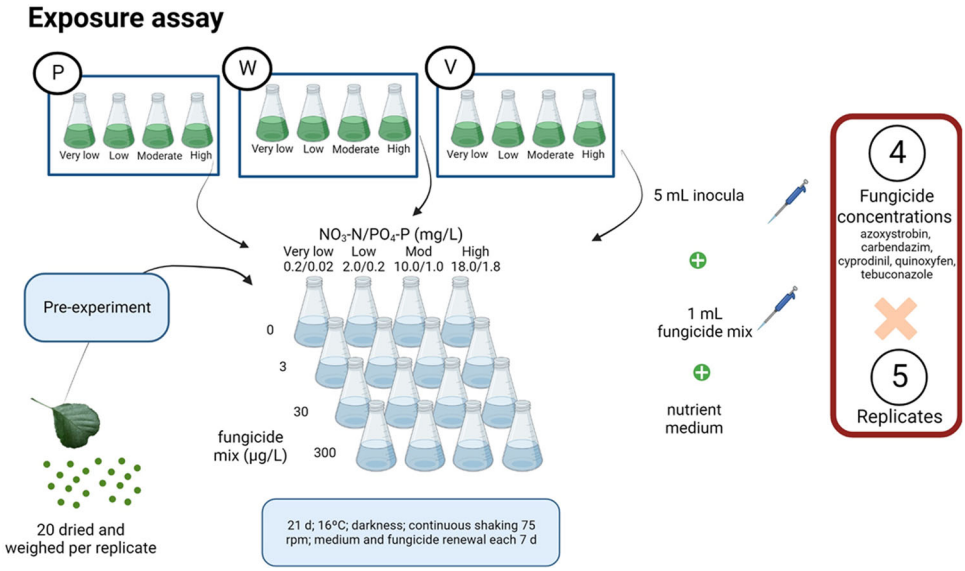

FIGURE 3: Exposure assay. The inocula prepared were used to microbially colonize leaf discs in Erlenmeyer flasks, while being exposed to increasing concentrations of nutrients and fungicides over 21 days, with media and fungicides being renewed every 7 days. Created with BioRender.com.

damage and infection (excluded), and then separated by size and stored at –20 °C until use. Freezing may cause minor changes in leaf decomposition (Bärlocher, 1992; Boyero et al., 2016), which may require caution when extrapolating findings or comparing with other studies that employed a different approach (e.g., air drying). After field colonization, the leaf material was transported to the laboratory in stream water. In the laboratory, invertebrates and sediment particles were carefully removed from leaves under running tap water. This cleaning step can potentially change the microbial assemblage, but it is the same for all replicates and necessary to avoid any confounding effect of invertebrate feeding. The latter could well over-ride changes in the microbial leaf decomposition activity driven by fungicide exposure. The inoculum from each sampling site was subsequently placed in an individual stainless-steel container (120 × 30 × 20 cm; volume 50 L) filled with 25 L of constantly aerated stream water from the respective sampling sites at 16 ± 1 °C in the dark for 7 days. In addition, another approximately 500 uncolonized black alder leaves were added to increase habitat diversity (i.e., different successional stages of leaves), supporting the maintenance of a diverse microbial community (Gessner et al., 1993).

Chemicals

The fungicide mixture consisted of five active ingredients, namely, azoxystrobin, carbendazim, cyprodinil, quinoxyfen, and tebuconazole, contained in pesticide formulations commonly applied in the region (Landesamt für Umwelt, 2016). The modes of toxic action, active ingredients, and respective manufacturers of the fungicide formulations are presented in the Supporting Information, Table S2. Sum nominal concentrations were 0 (control), 3, 30 (environmentally relevant concentrations), and 300 µg/L (high contamination). To confirm nominal concentrations of the individual fungicides, samples were taken from the test Erlenmeyer flasks approximately 2 h after test initiation as well as just before the weekly medium exchange (see the *Exposure assay* section) and analyzed using liquid chromatography- high resolution mass spectrometry (Thermo Fisher Scientific) following published protocols (as in Fernández et al., 2014; Supporting Information, S3, A.2.1). Although measured sum concentrations deviated partly by up to 30% from the nominal levels (Supporting Information, Table S3), mainly due to insufficient quantification limits (3 µg/L) or potential fungicide attachment to leaf material, the spacing factor between tested concentrations was reached, justifying the use of nominal concentrations in the following procedures.

Exposure assay

Prior to test initiation, leaf discs (diameter 20 mm) were cut from frozen and uncolonized leaves, randomly pooled in groups of 20 representing one replicate, dried at 60 °C for 24 h, and weighed together to the nearest 0.01 mg. Forty-eight hours before the initiation of each bioassay, dried and preweighed leaf discs were leached in autoclaved nutrient medium with treatment-matched nutrient concentrations to avoid any potentially confounding impact on microbial leaf decomposition (Bärlocher, 2020). Five additional replicates/nutrient concentration were included, which were used to correct for additional leaching-induced and physical leaf mass loss. Futhermore, 9.9 g wet weight of leaf material from the stainless-steel containers (see above) were transferred to 150 mL of nutrient medium with treatment-matched nutrient levels and homogenized on ice using an Ultra-Turrax® T25 (IKA®-Werke) to generate microbial inocula suspensions. Subsequently, 5 mL of these suspensions, 20 preweighed and leached leaf discs, and 1 mL of fungicide stock solution were transferred into sterilized 150-mL Erlenmeyer

flasks, and autoclaved nutrient medium was added to reach a final volume of 50 mL. Erlenmeyer flasks were closed with sterile culture cellucotton plugs allowing air exchange, kept at 16 ± 1 °C in the dark under continuous orbital shaking at 75 rpm, and the nutrient medium together with the fungicide mixture was renewed every 7 days. After 21 days, the bioassay was terminated and leaf discs were recovered and separated. From the 20 leaf discs, two randomly chosen leaf discs were used to characterize the leaf-associated microbial communities, and one leaf disc was used to quantify exoenzyme activities. For these purposes, leaf discs were lyophilized and weighed to the nearest 0.01 mg. The dry weight of the remaining 17 discs (dried at 60 °C for 24 h and weighed to the nearest 0.01 mg) was used to estimate the final dry weight of the 20 discs (final weight = (dry wt/17) × 20) and quantify the microbially mediated decomposition rates (see the *Data analysis* section for details; Benfield, 2007).

Exoenzyme activity

Hydrolase and oxidase activities were quantified using the method described by DeForest (2009) but modified for its use to analyze leaf litter (see Baudy et al., 2021). Detailed information is provided in the Supporting Information, SA.2.2. Enzymatic activities were expressed as μmoL of degraded substrate/mg leaf dry weight/h (DeForest, 2009). Subsequently, the data were used to calculate the recalcitrance ratio of the leaf material as normalized oxidases/total hydrolases activities (Supporting Information, Table S4). The higher the ratio of oxidase to hydrolase activities, the greater the relative investment for degradation of recalcitrant carbon is (Romero-Olivares et al., 2017).

Characterization of leaf-associated microbial communities

Fungal and bacterial abundances. The FastDNA® Spin Kit for Soil in combination with the FastPrep™-24 5G Instrument (MP Biomedicals) was used to extract DNA from leaf material. In addition, we processed empty extraction tubes as negative controls in each extraction run. The amounts of fungal and bacterial operon copies were quantified as proxies for overall leaf-associated fungal and bacterial abundances, respectively, via SYBR® Green reactions (Manerkar et al., 2008). The real-time PCR solutions (total of 10 μL) consisted of 2.8 μL of DNAse-free water, 0.1 μL of forward primer, 0.1 μL of reverse primer (both at 10 μmoL/μL, from biomers.net; see more details in the Supporting Information, Table S5), 2 μL of 50-fold diluted DNA extract, and 5 μL of PowerUp™ SYBR® Green Master Mix (Applied Biosystems). The PCR cycling conditions consisted of initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s, and extension at 72 °C for 60 s. At the end of each run, a melting curve analysis was performed to ensure the specificity of the assays. Real-time PCRs were performed on a Mastercycler® ep gradient S (Eppendorf) using 0.2-mL 8-tube strips covered with clear optical 8-cap strips (Sarstedt). The results were dry weight normalized to the respective leaf discs. Further details on the assays are provided in the Supporting Information, Table S5.

Fungal community composition. The DNA extracts (described in the previous paragraph) were used to perform NGS according to the protocol of Carl et al. (2022). For each of the studied communities (P1–3, V1 and 2, and W1–3), three levels of fungicides (0, 30, and 300 μg/L, excluding 3 μg/L) and nutrients (very low, low, and high) were evaluated, omitting the low fungicides and medium nutrient concentrations, respectively. This narrowed focus was motivated by the expected effects at higher fungicide concentrations and the fact that these nutrient concentrations reflect the range reported for the sampling sites (Supporting Information, Table S1) or excess of nutrients compared with sampling sites (high concentration).

Preparation of leaf samples for sequencing on the Illumina MiSeq device is described in Carl et al. (2022), with detailed information provided in the Supporting Information, SA.2.3. Amplicon libraries of the fungal ITS2 rDNA gene were generated using a mix of five forward primers (ITS3tagmix) and one reverse primer (ITS4ngs;Tedersoo et al., 2014, 2015). The PCR products were pooled for each sample to account for the technical bias of PCR reactions (Lindahl et al., 2013). For metabarcoding, barcodes, sequencing adaptors, and indices were ligated to the products of the first PCR. The resulting ITS2 library was sequenced on the Illumina MiSeq System using the chemistry of a 600-cycle MiSeq Reagent Kit Ver. 3 (Illumina). Indices were demultiplexed, followed by barcode demultiplexing using an in-house script from the Leibniz Institute German Collection of Microorganisms and Cell Cultures (DSMZ; GitHub, 2023a) Sequences were processed with PIPITS (Ver. 2.4;, Gweon et al., 2015; GitHub, 2023b). Taxonomic assignment was performed using the trained data sets of the Ribosomal Database Project classifier (UNITE DB Ver. February 02, 2019). From this, PIPITS created an operational taxonomic unit (OTU) table for every sample, which was assigned according to the "Species Hypothesis" of the UNITE database (Nilsson et al., 2019). Classification of OTUs was curated as described in Carl et al. (2022). In brief, (1) classification assigned to OTUs was reblasted against the US National Center for Biotechnology Information reference databases (nucleotide collection of GenBank BLAST®; megablast within "blastn" web application; National Institutes of Health, 2023; (2) the classification was corrected, if necessary, as detailed in Carl et al. (2022); and (3) OTUs assigned to the same species hypothesis were merged to one taxon to lessen the marker bias of the internal transcribed space (ITS) region, and OTUs leading to the same species curation were merged/sample. The criteria used for the curation of each OTU were: (1) significant similarity to any BLAST-hit of a fungal taxon (≥95%); (2) reasonable coverage of sequence (≥95%); (3) highest e-value (ratio between coverage and similarity of the sequence); and (4) reliably published sequence (reference database, isolate voucher, publication yes/no) of the fungal ITS rDNA region (Heeger et al., 2018; Supporting Information, Table S7). Within the whole data set, 178 taxa passed our quality criteria. From these 178 taxa, those appearing only once were excluded from

further analysis to reduce random noise; this procedure did not influence the overall outcome of our analyses. The remaining 93 taxa were used to characterize the fungal community in each treatment (Supporting Information, Tables S6 and S7).

Data analysis

The variables "exposure history" and "season" (time of the sampling) were highly correlated (multicollinearity); thus "season" was excluded from further analysis because our study was designed to focus on "exposure history." Data obtained from microbial inocula collected from sampling sites with the same land use were used as replicates for data analysis. This pooling approach allowed us to generalize the findings and draw more robust conclusions about the microbial communities from P-, W-, and V-streams and their responses to the experimental conditions. Microbially mediated leaf decomposition rates, expressed as $k_{microbial}$ (d^{-1}), were calculated as follows, according to Benfield (2007):

$$k_{microbial} = \frac{-\ln(dwf/(dwi \times l))}{t}$$

where *dwf* and *dwi* refer to the final and the initial dry weights of leaf discs, *l* is a dimensionless empirical factor used to correct for the leaf mass loss due to leaching, with leaching being dependent on the treatment (ranging in the present study between 0.74 and 0.81), and *t* is the decomposition time (21 days). Subsequently, we fitted dose–response models ("drm"-command) on the leaf decomposition rates of each exposure history and nutrient level against fungicide concentrations. The best fitting models (always lower limit at 0) were chosen based on visual judgment and Akaike's information criterion (see the Supporting Information, Table S8, for detailed information).

Shapiro–Wilk and Levene's tests were used to test for normality of residuals and homoscedasticity of univariate data (all data except fungal community composition). If the assumptions for parametric testing were met (which was only the case for for enzyme activity), analyses were run on the original data by applying three-factor analyses of variance (ANOVAs) with the independent variables, exposure history ("history"), fungicide exposure ("fungicide"), and nutrient concentration ("nutrient"), followed by post hoc comparisons for main effects with Bonferroni *p* value adjustment. Because the assumptions for parametric testing were violated for microbially mediated leaf decomposition as well as fungal and bacterial abundances, aligned rank transformation ANOVA tests were used instead. To simplify the comparisons and statistical testing, the very low nutrient level at 0 µg fungicides/L was set as the control for P-communities, whereas for W- and V-communities, the control was set at the low nutrient level and 0 µg fungicides/L. This distinction was made because W- and V-communities experienced higher nutrient levels in the field relative to P-communities (see the Supporting Information, Table S1).

For multivariate fungal community composition data, comparisons were conducted at the species level, a presence–absence table (1/0; Supporting Information, Table S7) was created, and nonmetric multidimensional scaling plots (NMDS; Clarke, 1993) were generated using the Jaccard coefficient. The assumption of homogeneous within-group dispersion was tested using the "betadisper" function within the R-package "vegan." Subsequently, a factorial permutational multivariate analysis of variance (Anderson et al., 2005) was performed on the original data with 999 permutations to assess the individual and combined effects of the independent variables ("history," "fungicide," and "nutrient"), applying the Jaccard coefficient (Real & Vargas, 1996) as a distance measure between groups. Statistics were conducted and figures were prepared using R Ver. 4.2.1 (R Core Team, 2022) as well as the add-on packages "vegan" (Oksanen et al., 2009), "ggplot2" and "ggh4x" (Wickham, 2016), "tidyr" (Wickham, Vaughan, et al., 2023), "dplyr" (Wickham, François, et al., 2023), "rstatix" (Alboukadel, 2023), "visreg" (Breheny & Burchett, 2017), and "ARTool" (Kay et al., 2021). Note that the term "significant(ly)" refers to statistical significance ($p < 0.05$) throughout our study.

RESULTS AND DISCUSSION

Contrary to our first hypothesis, elevated fungicide concentrations ($p > 0.05$; Figure 4 and Table 1) did not affect microbially mediated leaf decomposition. Instead, P- and W-communities may have benefitted from fungicide exposure at 30 and 300 µg/L (Supporting Information, Figure S1), observed as nonsignificant 30% increases in leaf decomposition rates compared with the respective fungicide-free controls (Supporting Information, Table S10). The effect of fungicides was not reflected in the microbial communities' relative investment in degrading recalcitrant carbon (i.e., the recalcitrance ratio; Supporting Information, Table S4), which was not significantly affected by the factors "history" and "fungicide" ($p > 0.4$; Table 1 and Supporting Information, Table S10), contrary to our second hypothesis. In support of our third and partially contradicting our fourth hypotheses, elevated levels of nutrients tended to be a buffer for the nonsignificant fungicide-induced effects on leaf decomposition compared with fungicide-free treatments (Supporting Information, Figure S1 and Table S10). Additionally, fungal community composition was significantly changed by elevated fungicide concentrations (see the following section). However, changes in the fungal community structure seemed to be decoupled from its function, represented by leaf decomposition (see Feckler & Bundschuh, 2020).

Effects of fungicides on microbial communities with differing exposure histories

In addition to the effects on leaf decomposition of communities from P- and W-streams, fungicides had significant effects on the leaf-associated microbial community structure, namely, on bacterial and fungal abundances (both $p < 0.01$; Table 1), which have also been reported elsewhere (see Feckler et al., 2018; Fernández et al., 2015). The bacterial and fungal abundances showed no significant changes at low-to-intermediate fungicide concentrations (3 and 30 µg/L; Table 1

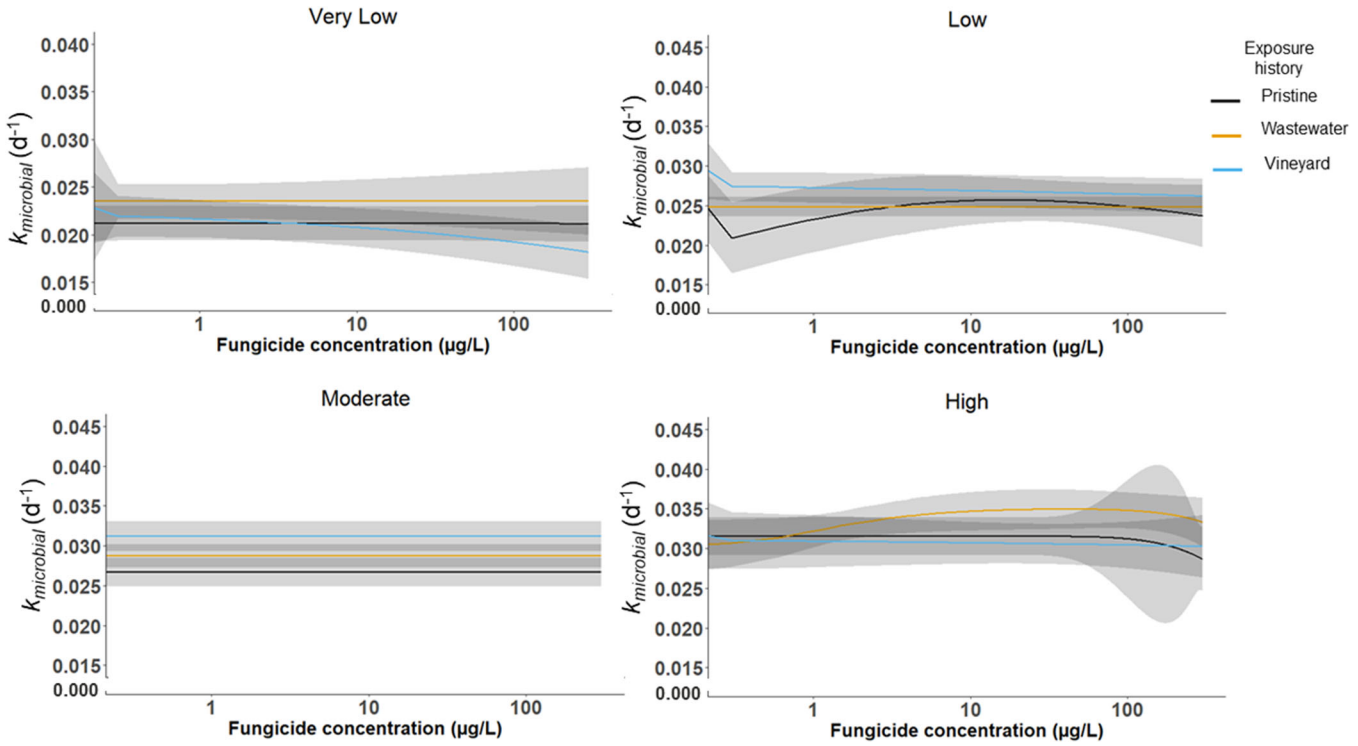

FIGURE 4: Dose–response models for the microbial breakdown rate ($k_{microbial}$ (d^{-1})) as a function of the total fungicide concentration (log10 scale), displayed separately for the four different nutrient levels. Shaded lines indicating corresponding 95% confidence bands ($n = 5$).

and Supporting Information, Figures S5 and S6 and Tables S1 and S12; $p < 0.05$) compared with the respective controls, whereas in Fernández et al. (2015), bacterial density tended to increase in vineyard-impacted sites. However, across all fungicide concentrations, the abundances were consistently lower in the V-community compared with the equivalent treatment in the W- and P-communities (Table 1 and Supporting Information, Tables S10, S11, and S12). Moreover, the high fungicide concentration (300 µg/L) affected fungal abundances independent of the history or nutrient level ($p < 0.05$; Table 1 and Supporting Information, Tables S11 & S12 and Figure S6).

Fungal communities of the control and lower fungicide concentrations (0 and 3 µg/L) showed considerable similarity in terms of species composition, whereas a substantial difference relative to the highest fungicide concentration was uncovered—a pattern observed across all nutrient levels ($p = 0.001$; Figure 5). The same pattern among fungicide concentrations was also reported in terms of fungal taxa richness (Supporting Information, Figures S7, S8, and S9). Moreover, fungal community composition differed among exposure histories ($p = 0.001$; Table 1). Thus these observations partially contradict the hypothesized link between the fungal community structure and their function (fourth hypothesis), because we expected to see an effect on the function leaf decomposition based on the diversity and abundance changes of the fungal species within the community. Our results are pointing toward functional stability despite community shifts (reviewed in Feckler & Bundschuh, 2020). Functional stability could be achieved due to functional similarity (Eisenhauer et al., 2023) within microbial communities and an increase in the dominance of tolerant fungal species that are at the same time more efficient in leaf decomposition (Ferreira & Chauvet, 2012; Pascoal et al., 2005). This assumption is supported by the NGS data, because in most of the cases tolerant AH species of the genus *Tetracladium* (*T. marchalianum*, *T. breve*, and *T. setigerum*) with a superior leaf decomposition efficiency (see Andrade et al., 2016; Duarte et al., 2006; Zubrod, Feckler, et al., 2015) dominated at high fungicide exposure independent of exposure history (Supporting Information, Table S7). Besides the increasing relevance of the genus *Tetracladium*, the species *Lemonniera terrestris*, *Flagellospora curvula*, and *Fusarium oxysporum* were more frequently detected with elevated fungicide concentrations. Although those species are considered to be tolerant, knowledge of their traits is limited and partly contradictory, hampering a mechanistic interpretation (Bundschuh et al., 2011; Pascoal et al., 2005). Nonetheless, Bundschuh et al. (2011) found *F. curvula* to be most abundant under control conditions, with a decreasing appearance at higher fungicide concentrations. In contrast, we found this species most frequently in the presence of fungicides. The opposite pattern has been observed for *Clavariopsis aquatica*: Pascoal et al. (2005) frequently detected this species in polluted streams of Northern Portugal, whereas we found this species more frequently in the absence of fungicides, suggesting phenotypic plasticity (see Quainoo et al., 2016). Notwithstanding, our findings support the principle of stable functioning being mediated by the dominance of highly efficient decomposers. These results are supported by other studies (reviewed in Feckler & Bundschuh, 2020) pointing to a stable functional performance (i.e., leaf decomposition) when the microbial community is dominated by a few species

TABLE 1: Output for statistical analyses, namely, aligned ranks transformation analysis of variance (ANOVA) for microbial leaf decomposition as well as bacterial and fungal abundance[a], ANOVA for recalcitrance ratio, and factorial permutational multivariate ANOVA for fungal community composition

Variable	Source of variation	*Df*	SS	*Df* res	*F* value	*p* Value
Leaf decomposition	Fungicide	3	—	592	0.367	0.776
	Nutrient	**3**	—	**592**	**70.938**	**<0.001**
	History	**2**	—	**592**	**6.592**	**0.001**
	Fungicide × Nutrient	9	—	592	1.446	0.164
	Fungicide × History	6	—	592	1.151	0.330
	Nutrient × History	**6**	—	**592**	**3.100**	**0.005**
	Fungicide × Nutrient × History	18	—	592	0.268	0.999
Bacterial abundance	**Fungicide**	**3**	—	**336**	**8.204**	**<0.001**
	Nutrient	3	—	336	1.839	0.139
	History	**2**	—	**336**	**4.009**	**0.019**
	Fungicide × Nutrient	9	—	336	0.854	0.566
	Fungicide × History	6	—	336	0.202	0.975
	Nutrient × History	**6**	—	**336**	**3.059**	**0.006**
	Fungicide × Nutrient × History	18	—	336	1.186	0.269
Fungal abundance	**Fungicide**	**3**	—	**336**	**7.499**	**<0.001**
	Nutrient	3	—	336	1.888	0.131
	History	**2**	—	**336**	**3.089**	**0.046**
	Fungicide × Nutrient	9	—	336	1.013	0.428
	Fungicide × History	6	—	336	0.234	0.965
	Nutrient × History	**6**	—	**336**	**4.255**	**<0.001**
	Fungicide × Nutrient × History	18	—	336	1.318	0.173
Recalcitrance ratio	Fungicide	1	<0.001	<0.001	0.003	0.958
	Nutrient	3	<0.001	<0.001	0.483	0.697
	History	2	<0.001	<0.001	0.943	0.403
	Fungicide × History	2	<0.001	<0.001	0.560	0.579
	Nutrient × History	6	<0.001	<0.001	0.231	0.962
	Fungicide × Nutrient	3	<0.001	<0.001	0.164	0.919
	Fungicide × Nutrient × History	6	<0.001	<0.001	0.324	0.918
	Residuals	24	0.002	<0.001		
Community composition	**Fungicide**	**1**	**2.738**	**0.127**	**11.145**	**0.001**
	Nutrient	**2**	**0.711**	**0.033**	**1.447**	**0.034**
	History	**2**	**1.340**	**0.062**	**2.728**	**0.001**
	Fungicide × Nutrient	2	0.571	0.026	1.163	0.208
	Fungicide × History	**2**	**0.753**	**0.035**	**1.533**	**0.018**
	Nutrient × History	4	1.105	0.051	1.124	0.197
	Fungicide × Nutrient × History	4	0.974	0.045	0.991	0.485
	Residual	54	13.267	0.618		
	Total	71	21.462	1		

[a]See the respective post hoc testing values in the Supporting Information, Table S11).
p values printed in bold indicate statistical significance.
Df = degrees of freedom; Df res = residual degrees of freedom for each model; *F* value = ratio of variances; SE = standard error of the estimate; SS = sum of squares.

with superior traits that compensate for biodiversity loss (Dangles & Malmqvist, 2004).

Effects of nutrients on microbial communities with differing exposure histories

Leaf decomposition significantly benefited from elevated nutrient concentrations (our third hypothesis), whereas the effect strength depended on the exposure history ($p = 0.005$; Table 1). Especially at moderate and high nutrient levels, leaf decomposition increased by 15% to 32%, 11% to 18%, and 3% to 7% for the P-, W-, and V-communities (Supporting Information, Table S10), respectively, relative to the respective control scenarios in the absence of fungicides (Figure 4 and Supporting Information, Table S10). These observations may be explained by the dynamic energy budget theory (Kooijman, 2000), namely, that the ease of accessing nutrients from the medium supports microbial growth and thus the functional performance because more energy is available for producing the exoenzymes needed for leaf degradation (Bärlocher & Corkum, 2003). This assumption is also supported by Feckler et al. (2018), who studied equivalents to the P- and V-communities that we assessed, and observed higher leaf decomposition in treatments with higher nutrient availability (see also Pascoal & Cássio, 2004; Suberkropp et al., 2010). Thus we assume that in ecosystems with higher nutrient inputs, chemical stressors have less impact on functioning due to "free" energy from the available nutrients (see Rossi et al., 2018 but also see Fernández et al., 2016).

Despite the positive effect of nutrients on leaf decomposition, microbial abundances were significantly affected by exposure history, with P-communities being characterized on average by 150% higher bacterial and fungal abundances compared with W- and V-communities within the same nutrient level (Supporting Information, Tables S10, S11, S12 and

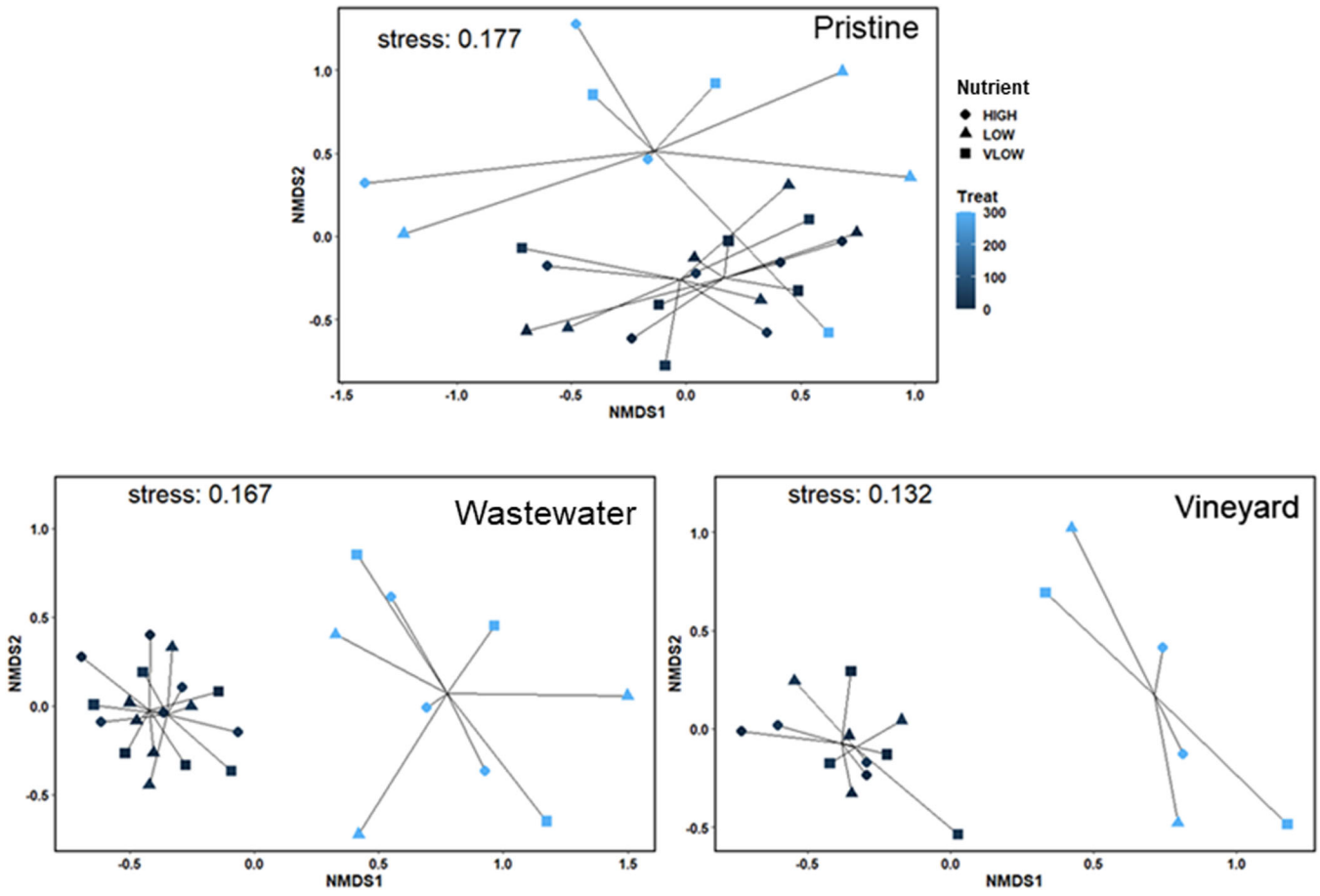

FIGURE 5: Nonmetric multidimensional scaling (NMDS) plots for leaf-associated aquatic hyphomycete communities originating from streams with differing land use in their catchments (Pristine, Wastewater treatment plants, Vineyard). Nutrient levels are indicated by symbols: very low = squares; low = triangles; high = circles. Colors indicate fungicide concentrations: 0 and 30 μg/L = dark blue, 300 μg/L = light blue. Spider webs connect the samples of each treatment at their respective group centroid. The stress value is provided as a measure of goodness-of-fit for NMDS, with a reasonable fit indicated when below 0.2 (Clarke, 1993).

Figures S5 and S6). Contrary to the structural parameters, the leaf decomposition performed by W-communities was slightly (up to 15%) but significantly ($p < 0.003$) higher in comparison with the P-communities, whereas in V-communities the function was up to 40% significantly lower than in P-communities ($p < 0.01$; Table 1 and Figure 4 and Supporting Information, Table S10). This observation may be an experimental artefact because the proxies used for bacterial and fungal abundances do not account for changes in the fungal community composition and consequently its functional traits (Englert et al., 2015; Rossi et al., 2018). It may be that microbes characterized by a high leaf decomposition efficiency and capable of maintaining the function dominate over those with a lower efficiency (see Reiss et al., 2010).

Combining chemical stressors and exposure history

Our study found changes in community structures at high fungicide exposure across all exposure histories. We expected more pronounced effects of fungicides on P-communities compared with communities with exposure history (W- and V-communities). This expectation was not met, potentially due to the presence of some tolerant species, such as *T. marchalianum*, also found in P-communities. The latter could also have happened due to the relatively low fungicide concentrations we used compared with other studies. Although sum fungicide concentrations of 300 μg/L exceed concentrations detected in the environment (e.g., sum pesticide concentrations measured during rainfall events went up to 83.4 μg/L in Bereswill et al., 2012), these levels (i.e., 300 μg/L) have been too low to cause more pronounced responses in leaf decomposition and community structure during laboratory studies (see Feckler et al., 2018; Gonçalves et al., 2023; Zubrod, Feckler, et al., 2015). Under field conditions, however, lower concentrations of fungicides contributed to changes in the fungal community structure (see Fernández et al., 2016). Moreover, the high variability and nonconsistent patterns found among our three bioassays could be explained by the different sampling season and the respective naturally differing enzyme activities (Bastias et al., 2022). The latter theory suggests that the local community and potentially the colonization dynamics play a significant role, which should be further studied (Mora-Gómez et al., 2016).

CONCLUSIONS

Overall, the present study shows that leaf decomposition was not affected by elevated fungicide concentrations, and "fungicides" or "history" did not affect the degradation of recalcitrant carbon by microbial communities. Nonetheless, elevated fungicide concentrations significantly changed the fungal community composition across all exposure histories. The changes in the fungal community composition seemed to be decoupled from its function, represented by leaf decomposition, which points toward functional stability despite community shifts (Feckler & Bundschuh, 2020). The changes in fungal composition in the present and previous studies suggest phenotypic plasticity and support the principle of stable functioning being mediated by the dominance of highly efficient decomposers. These tolerant decomposer species with superior traits maintain functional performance while compensating for biodiversity loss (Dangles & Malmqvist, 2004).

Our study points to the benefits of jointly assessing ecosystem structure and function, which not only supports the interpretation of the data but also fuels biodiversity—ecosystem functioning research—particularly in the context of chemical stressors. The changes in the fungal community composition under fungicide exposure despite functional stability raises potential concerns: first, in case only functional measures (here approximated by leaf decomposition) are used to assess environmental impacts of stressors, structural changes will remain unnoticed; second, even if the function is stable, structural changes can potentially have wider effects because aquatic fungi play a key role in regulating aquatic food webs from the bottom up (Arsuffi & Suberkropp, 1989; Gonçalves et al., 2014). The latter may indeed be of concern because fungal species considered tolerant are often less nutritional for shedders than their sensitive counterparts, affecting the fitness and development of shredders (see Gonçalves et al., 2024). To advance our mechanistic understanding of this bottom-up regulation, the consideration of fungal traits under multiple stress scenarios may be beneficial (Loreau et al., 2001).

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5863.

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