Environmentally relevant fungicide levels modify fungal community composition and interactions but not functioning[☆]

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ABSTRACT

Aquatic hyphomycetes (AHs), a group of saprotrophic fungi adapted to submerged leaf litter, play key functional roles in stream ecosystems as decomposers and food source for higher trophic levels. Fungicides, controlling fungal pathogens, target evolutionary conserved molecular processes in fungi and contaminate streams via their use in agricultural and urban landscapes. Thus fungicides pose a risk to AHs and the functions they provide. To investigate the impacts of fungicide exposure on the composition and functioning of AH communities, we exposed four AH species in monocultures and mixed cultures to increasing fungicide concentrations (0, 5, 50, 500, and 2500 μg/L). We assessed the biomass of each species via quantitative real-time PCR. Moreover, leaf decomposition was investigated. In monocultures, none of the species was affected at environmentally relevant fungicide levels (5 and 50 μg/L). The two most tolerant species were able to colonize and decompose leaves even at very high fungicide levels (≥500 μg/L), although less efficiently. In mixed cultures, changes in leaf decomposition reflected the response pattern of the species most tolerant in monocultures. Accordingly, the decomposition process may be safeguarded by tolerant species in combination with functional redundancy. In all fungicide treatments, however, sensitive species were displaced and interactions between fungi changed from complementarity to competition. As AH community composition determines leaves' nutritional quality for consumers, the data suggest that fungicide exposures rather induce bottom-up effects in food webs than impairments in leaf decomposition.

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1. Introduction

Aquatic fungi adapted to colonize and degrade submerged leaf litter (mainly aquatic hyphomycetes; = AHs; Baschien et al., 2013; Dighton and White, 2017) are key to ecosystems fueled by allochthonous organic matter. Making use of a diverse set of extracellular enzymes, AHs efficiently catalyze leaf litter decomposition, thereby converting non-utilizable biopolymers to bioavailable substances (Evans and Hedger, 2001). Through the simultaneous accumulation of fungal biomass, they additionally increase leaves' nutritional quality (i.e., conditioning; Cummins and Klug, 1979) for consumers, such as shredders.

Globally, the majority of streams are subject to human impact, including pollution with xenobiotics (Vörösmarty et al., 2010). Synthetic fungicides represent a group of xenobiotics applied to control fungal pathogens and enter streams following their use in agricultural and urban landscapes (as reviewed by Zubrod et al., 2019). Since fungicides target evolutionary conserved molecular processes of close relatives to AHs (Stenersen, 2004), these compounds pose a risk for aquatic fungi and the functions they provide. Moreover, an overlap of measured field and effect concentrations of fungicides, which is particularly large for the groups of demethylation inhibitors and strobilurins (e.g., tebuconazole and

azoxystrobin, respectively; Zubrod et al., 2019), causes further concern.

Changes in the functioning of AH communities, mostly assessed via leaf mass loss or total fungal biomass, are predominantly induced at high fungicide concentrations (Pimentão et al., 2020; Zubrod et al., 2019). In contrast, structural changes (i.e., diversity or community composition) may already appear at low (field relevant) concentrations (Fernández et al., 2015; Pimentão et al., 2020; Zubrod et al., 2019). This discrepancy may be explained by the replacement of sensitive by tolerant AH species in combination with functional redundancy (Pascoal et al., 2005). While ecological mechanisms (e.g., selection or complementarity effects) linking biodiversity and ecosystem functioning (i.e., B-EF relationships) in AH communities remain poorly understood (Bärlocher, 2016; Grossart and Rojas-Jimenez, 2016), it is assumed that resource partitioning constitutes a major type of interaction in undisturbed communities (e.g., via complementary extracellular enzyme inventories; Gessner et al., 2010). Assessing impacts of fungicide exposures on B-EF relationships requires the ability to track the biomass of individual AH species within a community throughout various successional stages, which is offered by species-specific quantitative real-time PCR (qPCR; Duarte et al., 2006; Grossart and Rojas-Jimenez, 2016). To the best of our knowledge, only one study exists that applied species-specific qPCR assays to assess B-EF relationships in AH assemblages under chemical stress (i.e., Fernandes et al., 2011). In this study, cadmium exposure altered the relative abundance of AH species and induced shifts from complementary towards competitive interactions, while leaf decomposition was maintained by cadmium-tolerant strains due to dominance effects (i.e., selective pressure of cadmium favored tolerant species at the expense of others).

Using a similar experimental setup and novel species-specific TaqMan® qPCR assays (Baudy et al., 2019), the present study aimed at investigating B-EF relationships in AH communities under fungicide exposure. Employing a factorial microcosm experiment, leaves were colonized by four AH monocultures, six binary, and one quaternary combination (i.e., eleven different cultures). Each culture was exposed to five concentrations of a five-component fungicide mixture, reflecting environmentally relevant (i.e., chronic exposure to fungicide mixtures in the low μg/L range) to worst-case exposure scenarios (exposure in the mg/L range; Zubrod et al., 2019). To assess the functional performance of these communities, leaf mass loss, fungal biomass production (via ergosterol), and the activity of seven extracellular enzymes were analyzed. AH community composition was investigated via species-specific qPCR (Baudy et al., 2019).

We expected that the AH species (in monocultures) differ in their fungicide tolerance, as has been reported for aquatic fungi exposed to different concentrations of various fungicides (Dijksterhuis et al., 2011). We additionally hypothesized that cultures would be more resistant with increasing diversity and maintain leaf decomposition up to high fungicide concentrations, due to functional redundancy and the presence of tolerant species (Biggs et al., 2020; Pascoal et al., 2005). Community composition, on the other hand, would already be affected at relatively low concentrations (Zubrod et al., 2019). We finally hypothesized that fungicide exposure alters the interactions between AH species (e.g., complementarity and dominance effects) occurring during leaf colonization (cf. Fernandes et al., 2011), which would be most pronounced in assemblages comprising species with marked differences in fungicide tolerance.

2. Material and methods

2.1. Chemicals

The fungicide mixture comprised the synthetic fungicides azoxystrobin, carbendazim, cyprodinil, quinoxyfen, and tebuconazole applied at equal concentrations. These fungicides cover a broad range of modes of action (Table 1) currently used in agriculture and were already detected in the same streams within one year (Landesamt für Umwelt Rheinland-Pfalz, 2016). The fungicide sum concentrations of 0, 5, 50, 500, and 2500 μg/L were chosen based on an earlier study (Zubrod et al., 2015a), and to cover field-relevant (i.e., 5 and 50 μg/L) to worst-case exposure scenarios (i.e., 500 and 2500 μg/L; Rabiet et al., 2010; Zubrod et al., 2019). Fungicides were applied as commercial products (Table 1), making the use of further solvents redundant. The products were diluted in autoclaved (at 121 °C for 20 min; Systec DE-65®, Systec, Germany) nutrient medium (for composition see Dang et al., 2005) to obtain the respective nominal sum concentrations. To verify exposure concentrations, volumes of 10 mL were sampled in the 0, 5, and 2500 μg/L fungicide treatments ($n = 1/3/3$) at test initiation and at the time of the first medium renewal (i.e., after 7 days). Samples were taken from fresh medium in additionally prepared microcosms at test initiation and old medium of quaternary cultures at the time of medium renewal on day 7 of the experiment. Samples were stored frozen at −20 °C until chemical analysis. After thawing, fungicides were measured by direct injection into a liquid chromatography high-resolution mass-spectrometry (LC-HRMS) Orbitrap system (Thermo Fisher Scientific, Germany) using matrix-matched standards for calibration. The limit of quantification (LOQ) was set to the lowest concentration reliably distinguishable from blanks and ranged from 0.1 to 0.5 μg/L among fungicides (Table 1; for details see Fernández et al., 2016). Fungicide concentrations in the controls were below the LOQ. Due to technical reasons, concentrations of quinoxyfen could not be quantified. However, as the measured sum concentrations of the other four fungicides deviated less than 15% of the nominal (Table 1), a proper dosing with all five fungicides at test initiation is assumed. Thus, nominal sum concentrations are used throughout the present manuscript.

2.2. Fungi and leaf substrate

Strains of the AH species *Alatospora acuminata*, *Heliscella stellata*, *Neonectria lugdunensis*, and *Tetracladium marchalianum* were used as model fungi (for strain specifications see Table S1). These species are representatives of decomposer communities in temperate streams and are assumed to vary in their tolerance to the fungicide mixture used in this study (Bundschuh et al., 2011; Zubrod et al., 2015a). The strains were isolated in 2015 and 2016 from small streams in Germany and deposited at the German Collection for Microorganisms and Cell Cultures (Leibniz-Institute DSMZ; Baudy et al., 2019). Cultures were grown on Petri dishes containing 15 mL of 1% malt extract agar (10 g/L malt extract, 20 g/L agar) at 16 °C in darkness for 21 days. Agar plugs (diameter: 5 mm) were cut from the growing edges of colonies and served as fungal inoculum.

Leaves of *Alnus glutinosa* (L.) GAERTN. (black alder), a wide-spread European riparian tree species (Copolovici et al., 2014), with no visible signs of damage or symptoms of diseases, were handpicked from trees near Landau, Germany (49.20116 °N; 8.09331 °E) shortly before abscission in 2015 and stored frozen at −20 °C (instead of

Table 1
Information on origin, mode of action and concentrations of the applied fungicides.

Fungicide	Product	Manufacturer	Mode of action[a]	Analytical limit of quantification (µg/L)	Fungicide concentrations (µg/L)					
					5 µg/L fungicide treatment			2500 µg/L fungicide treatment		
					Nominal	Measured in fresh nutrient medium[b] ($n = 3$)	Measured in old nutrient medium[b] ($n = 3$)	Nominal	Measured in fresh nutrient medium[b] ($n = 3$)	Measured in old nutrient medium[b] ($n = 3$)
Azoxystrobin	Ortiva®	Syngenta Agro GmbH, Germany	Inhibition of respiration	0.4	1	0.68 ± 0.03 (68.0%)	0.50 ± 0.06 (50.3%)	500	481.3 ± 58.8 (96.3%)	256.0 ± 22.3 (51.2%)
Carbendazim	Derosal®	Bayer Crop Science, Germany	Inhibition of mitosis and cell division	0.1	1	1.04 ± 0.30 (104.3%)	0.16 ± 0.07 (15.7%)	500	447.7 ± 78.0 (89.5%)	295.7 ± 34.6 (59.1%)
Cyprodinil	Chorus®	Syngenta Agro GmbH, Germany	Inhibition of amino acids and protein synthesis	0.1	1	0.84 ± 0.03 (84.0%)	< LOQ[c]	500	453.0 ± 67.4 (90.6%)	38.3 ± 5.7 (7.7%)
Quinoxyfen	Fortress™ 250	Dow AgroSciences GmbH, Germany	Inhibition of signal transduction	—	1	NA[d]	NA[d]	500	NA[d]	NA[d]
Tebuconazole	Folicur®	Bayer Crop Science, Germany	Inhibition of sterol biosynthesis in membranes	0.5	1	1.41 ± 0.34 (140.7%)	0.79 ± 0.01 (79.0%)	500	373.3 ± 49.4 (74.7%)	156.7 ± 10.1 (31.3%)
Mixture	All of the above	All of the above	All of the above	—	5	3.97 ± 0.36 (99.3%[e])	1.45 ± 0.01 (36.3%[e])	2500	1755.3 ± 250.9 (87.8%[e])	746.7 ± 67.6 (37.3%[e])

[a] Fungicide Resistance Action Committee (2020).
[b] Mean concentration and standard deviation (percent recovery from nominal concentration).
[c] Limit of quantification.
[d] Not analyzed.
[e] Recovery based on nominal sum concentrations adapted to the proportion of the measurable four fungicides (i.e., 4 and 2000 µg/L), excluding quinoxyfen.

dry storage) for practical reasons. It is noted that frozen storage can impact leaching and conditioning of leaves (Bärlocher, 1992), which does, however, not impede comparability across treatments (see below for details). Upon thawing, disks (diameter: 16 mm) were cut, excluding the midrib. In the test vessels (i.e., 100-mL Erlenmeyer flasks), 20 leaf disks were leached in 20 mL of autoclaved nutrient medium for 48 h. Subsequently (i.e., one day prior to the experiment), leachates were decanted, whereas flasks and leaf disks therein were autoclaved to eliminate unwanted microbial activity. The sterile test vessels were kept overnight in a sealed ethanol-disinfected box.

2.3. Experimental design

The microcosm experiment was employed as an 11 × 5-factorial design. Eleven fungal treatments — comprising four monocultures, six binary, and a quaternary combination of the four species — were exposed to five fungicide sum concentrations (i.e., 0, 5, 50, 500, and 2500 µg/L) leading to 55 treatments. Additionally, two sterile (fungus-free) treatments receiving either no fungicides or a fungicide sum concentration of 2500 µg/L were prepared. These treatments served as references for later analyses (see below for details). All 57 treatments were replicated five times ($n = 5$). Inoculation of the microcosms largely followed the methods described in Andrade et al. (2016). Microcosms were initiated under sterile conditions in a laminar flow cabinet (UV-treated for 30 min prior to use; NU-437-500 E; Nuaire, USA). Each microcosm consisted of a 100-mL Erlenmeyer flask containing 20 leaf disks, four agar plugs, and 60 mL of nutrient medium spiked with the respective fungicide concentration. Microcosms receiving one, two, or four species were equipped with four, two, or one cultivated agar plug(s) per species. Reference microcosms containing no fungi were equipped with four sterile agar plugs. The test vessels were closed with gas-permeable cellulose stoppers. Incubation of the microcosms was carried out on a horizontal shaker (model VKS 75 B control; Edmund Bühler GmbH, Germany) at 115 rpm at 16 °C in darkness.

The microcosms were randomly distributed on the shaker and shuffled daily to avoid location effects. After 7 days of incubation, the nutrient medium and the respective fungicide concentrations were renewed in all microcosms (cf. Andrade et al., 2016). After the total incubation time of 14 days, leaf disks from all microcosms were sampled for later analyses as follows: for analysis of enzyme activity, one leaf disk was preserved in a 12-mL plastic centrifuge tube and stored at −20 °C; for assessment of bacterial contamination (this was largely prevented; see Supporting Information), two leaf disks were preserved in 10 mL of a 2% formaldehyde/0.1% sodium pyrophosphate solution and stored at 4 °C; for potential analysis of sporulation, which was, however, not within the scope of this study, two leaf disks were agitated in deionized water for 96 h, fixed using formaldehyde (resulting in a 2% formaldehyde solution), and stored at room temperature; the remaining 15 leaf disks were preserved in pre-weighed 2-mL Eppendorf tubes, lyophilized, analyzed for dry mass to the nearest 0.01 mg and afterwards used for ergosterol and DNA analyses.

2.4. Enzyme activity analyses

Activities of hydrolytic and oxidative enzymes were assessed based on DeForest (2009) with a detailed overview of the modifications being highlighted in Baudy et al. (accepted). Briefly, leaf disks were homogenized in 350 mL of sterile nutrient medium using an Ultra-turrax® blender (24000 rpm for 30 s; IKA®-Werke GmbH & Co. KG, Germany). Using fluorescence- and absorbance-based reporter substrates, leaf homogenates were analyzed for activities of phosphatases (EC 3.1.3.1 and 3.1.3.2; targeting phosphate esters), α-1,4-glucosidase (EC 3.2.1.20; targeting starch and maltose), β-1,4-glucosidase (EC 3.2.1.21; targeting cellulose), cellobiohydrolase (EC 3.2.1.91; targeting cellulose), β-1,4-xylosidase (EC 3.2.1.37; targeting hemicellulose), peroxidase (EC 1.11.1.7; targeting lignin), and phenol oxidase (EC 1.10.3.2; targeting lignin). Reactions were performed in 96-well 300-µL well plates (Thermo Fisher Scientific, USA), which were incubated on a rotary shaker

(120 rpm; KS 15; Edmund Bühler GmbH, Germany) in darkness for approximately 1 h (hydrolases) or 2 h (oxidases). Fluorescence and absorbance were measured using a microplate reader (Infinite 200, Tecan Group, Switzerland). The remaining leaf homogenate of each sample (~340 mL) was filtered through pre-weighed glass fiber filters (GF/6; Whatman GmbH, Germany), which were subsequently dried at 60 °C for 24 h and weighed to the nearest 0.01 mg. The difference of the filter weights was used to normalize enzyme activity to leaf dry mass.

2.5. Ergosterol analysis

Total fungal biomass was estimated as the fungal-specific membrane molecule ergosterol as described in Gessner (2005), without any further conversion (e.g., the average conversion factor of 5.5 μg ergosterol/mg fungal dry mass; Gessner and Chauvet, 1993). Briefly, ergosterol was extracted from 30 to 50 mg of lyophilized leaf sample in 10 mL of alkaline methanol. Extracts were purified via solid-phase extraction (Sep-Pak® Vac RC tC18 500 mg sorbent, Waters, USA), and eluted in isopropanol. Ergosterol was finally quantified using a high-performance liquid chromatography (HPLC) system (1200 Series, Agilent Technologies, USA).

2.6. Fungal biomass quantification via DNA analysis

To estimate the biomass of individual fungi, DNA was extracted and species-specifically quantified (Baudy et al., 2019). Since the relationship between DNA concentrations and fungal dry mass is subjected to considerable interspecific variability (Baudy et al., 2019), DNA concentrations between species are not readily comparable. Briefly, genomic DNA was extracted from 25 to 50 mg of lyophilized leaf sample using the FastDNA® Spin Kit for Soil in conjunction with the FastPrep™-24 5G Instrument (MP Biomedicals, Germany). Extracted DNA amounts of individual species were quantified via TaqMan® qPCR reactions (Applied Biosystems, USA) performed in a Mastercycler® ep gradient S (Eppendorf, Germany).

2.7. Data analyses

Leaf mass loss (*L*; in percent) was calculated as follows:

$$L = \frac{\left(\overline{w}_{sterile} - w_{fungi} \right)}{\left(\overline{w}_{sterile} \right)} \times 100$$

where $w_{sterile}$ is the mean final dry mass of leaf disks in sterile reference microcosms and w_{fungi} is the final dry mass of leaf disks in individual microcosms containing fungi. Ergosterol and DNA concentrations (expressed as μg/g leaf dry mass, respectively) were calculated as described in Baudy et al. (2019). Enzyme activities (expressed as μmol/(h*g leaf dry mass); for calculation see Baudy et al. (accepted)) were normalized to pooled fungicide-free and fungicide-treated (2500 μg/L) sterile reference microcosms, if variables in both treatments were not significantly different (as judged by *t*-tests). Otherwise, each treatment was normalized individually. In this case, reference values for the fungicide sum concentrations of 5, 50, and 500 μg/L were interpolated from linear regression curves based on the available reference data. Predictions for leaf mass loss (P_{lml}) and ergosterol concentrations (P_{erg}) in mixed cultures were calculated as follows:

$$P_{lml/erg} = \sum_{i=1}^{n} F_{lml/erg \; species \; i} \times c_{DNA \; species \; i}$$

where $F_{lml/erg \; species \; i}$ is the mean specific leaf mass loss or mean specific ergosterol concentration (Table S1) of component species *i* in the mixed culture, $c_{DNA \; species \; i}$ is the DNA concentration of component species *i* in the mixed culture and *n* is the number of species in the mixed culture (i.e., 2 or 4).

Multiple comparisons of leaf mass loss, ergosterol and DNA concentrations, as well as diversity effects between fungicide-free controls and fungicide treatments were performed via analysis of variance (ANOVA) followed by Dunnett's tests. Comparisons between observed and predicted leaf mass loss and ergosterol concentrations were performed using paired *t*-tests. For multivariate analysis, enzyme activities were log(x+1) transformed and min-max normalized to decrease the discriminatory power of enzymes with high activities. To assess the effects of fungal diversity, species combination (nested within diversity) and fungicide exposure as well as their interactions on enzyme activity profiles, permutational multivariate analysis of variance (PERMANOVA) was performed on enzyme activity profiles of all cultures. Distances between enzyme activity profiles were calculated using Euclidean distance. For the visualization of dissimilarities of the enzyme activity profiles, the distance matrix was subjected to non-metric multidimensional scaling (NMDS). All statistics and figures were prepared using R version 3.5.2 (R Core Team, 2018) as well as the add-on packages "multcomp", "plotrix" and "vegan". The term "significant(ly)" is only used with regard to statistical significance (p < 0.05) throughout this study.

2.8. Diversity effects on leaf colonization

To assess interactions between AH species during the colonization of leaf substrate, diversity effects were calculated from species-specific DNA concentrations in monocultures and mixed cultures within the same fungicide treatments, applying a modified Price equation (Fox, 2005). The calculation of these effects (in percent) is based on the assumption that species perform in mixed cultures equally well as in monocultures (i.e., additivity). Calculated net diversity effects can be partitioned into three component effects comprising trait-independent complementarity, dominance, and trait-dependent complementarity.

Net diversity effects are deviations between observed DNA concentrations in mixed cultures and DNA concentrations predicted from monocultures, weighted by the initial proportion of inoculum of each species in the mixture. Trait-independent complementarity is positive if the majority of species show higher DNA concentrations in mixed culture than expected based on their performance in monoculture, indicating synergistic interactions. It is negative if the majority of species show the opposite response, thus indicating antagonistic interactions. Dominance provides insights into the dominance relation between species. Dominance is positive if species with high DNA concentrations in monoculture perform better in mixed cultures, but at the expense of species with lower DNA concentrations in monoculture. It is negative if species with low DNA concentrations in monoculture perform better in mixed cultures, but at the expense of other species. Trait-dependent complementarity is positive if species with high DNA concentrations in monocultures perform better in mixed cultures, but not at the expense of other species. It is negative if species with low DNA concentrations in monoculture perform better in mixed cultures, but not at the expense of other species (for more details see Fox, 2005).

3. Results and discussion

3.1. Responses of monocultures

Since virtually no leaf mass loss was observed for *A. acuminata* and *H. stellata* under control conditions (2.6% and −4.4%, respectively; Fig. 1a), potential effects of fungicide exposure on these species' degradative capacity could not be detected. In contrast, *N. lugdunensis* and *T. marchalianum* efficiently decomposed leaves (up to 25% in the control), while this functional variable was only for *N. lugdunensis* significantly affected at a fungicide sum concentration of 2500 μg/L (Fig. 1a). Generally, as indicated by ANCOVA (Table S2), among all monocultures and mixed cultures, there was a strong linear relationship between leaf mass loss and cumulative enzyme activities. This relationship was not significantly modified by fungicide exposure (Table S2), which generally has the potential to affect the substrate affinity of enzymes (Artigas et al., 2012). In line with earlier studies (e.g., Suberkropp et al., 1983; Abdel-Raheem and Shearer, 2002; Baudy et al., accepted), the degradative capacity of the investigated AH species seems to be based on distinct enzyme activity profiles (Fig. 2a−d). While *A. acuminata* and *T. marchalianum* show a similar profile, characterized by hydrolases (Fig. 2a, d), the profile of *N. lugdunensis* is characterized by oxidases (Fig. 2c). The profile of *H. stellata*, however, does not seem to include the investigated enzymes (Fig. 2b), which may be explained by the low biomass of this species observed in this experiment, resulting in lower productivity (Fig. 1b) or a different leaf colonization strategy (Baudy et al., accepted). Similar to leaf mass loss, fungicide exposure affected enzyme activity profiles only at high concentrations of 500 and 2500 μg/L (Fig. 2a−d), indicating no pronounced alterations in the regulation of the investigated enzymes at environmentally relevant fungicide concentrations.

Fungal biomass (independent whether assessed as ergosterol or DNA) of *A. acuminata* and *H. stellata* was maintained at constant levels up to a fungicide sum concentration of 50 μg/L. At higher fungicide concentrations, no or very low biomasses could be detected (Fig. 1b; Fig. 3a and b). Again, *N. lugdunensis* and *T. marchalianum* had measurable ergosterol contents, even at the highest fungicide sum concentration, which were, however, significantly lower compared to controls (−84% and −64%, respectively; Fig. 1b). This pattern was also reflected by DNA concentrations of *T. marchalianum* (Fig. 3d). In contrast, *N. lugdunensis* showed a substantial increase (up to 65%) in DNA levels at fungicide sum concentrations of 50 and 500 μg/L, while ergosterol remained constant (Fig. 1b; Fig. 3c), leading to an over-prediction of DNA-based ergosterol concentrations (Fig. 1b). This pattern is likely triggered by a direct effect of one fungicide of the mixture, namely tebuconazole, on the production of both biomarkers (Baudy et al., 2020). Such an effect can result in altered biomarker levels, not reflecting true mycelial biomass (Baudy et al., 2020). Nonetheless, *A. acuminata* and *H. stellata* can be considered as more sensitive compared to *N. lugdunensis* and *T. marchalianum*. This sensitivity pattern is in line with previous laboratory studies assessing AH species' productivity via morphological identification and quantification of asexual spores termed conidia (Bundschuh et al., 2011; Zubrod et al., 2015b). In these studies, it was observed that increasing fungicide concentrations reduced sporulation of *A. acuminata* and *H. stellata* and maintained or even increased sporulation of *N. lugdunensis* and *T. marchalianum* in near-natural decomposer communities.

In the field, *N. lugdunensis* and *T. marchalianum* frequently dominate AH communities inhabiting aquatic ecosystems severely contaminated with metals or xenobiotics (Solé et al., 2008; Sridhar et al., 2000, 2005). Therefore, and considering the stressors applied in this study specifically target fungi, the remarkable tolerance of

these species to fungicides can be assumed to be based on highly sophisticated detoxification systems. Detoxification processes in AHs have, however, mostly been investigated with respect to metals (Krauss et al., 2011) and increases in glutathione synthesis are suspected to play a major role in the metal tolerance of *N. lugdunensis* and *T. marchalianum* (Braha et al., 2007; Miersch et al., 2005). Yet, this detoxification pathway may also explain the tolerance of these species to synthetic fungicides, as glutathione is involved in the elimination of reactive oxygen species formatted upon xenobiotic action as well as in the conjugation of xenobiotics during phase II biotransformation reactions (Pócsi et al., 2004). Moreover, fungal ligninolytic enzymes (e.g., peroxidase and phenol oxidase) can metabolize aromatic xenobiotics (Harms et al., 2011), mitigating their toxicity (Artigas et al., 2017). However, as the most tolerant AH species of the present study had no elevated oxidase activities (i.e., *T. marchalianum*; Fig. 2d) this degradation pathway seems less relevant. To unravel the underlying biotransformation processes, the combination of "-omics" (e.g., a well-coordinated use of genomics, transcriptomics and proteomics; Tsui et al., 2016) and chemical analyses (internal vs. external concentrations of fungicides and their metabolites) is a promising approach.

Predictions of leaf mass loss based on DNA concentrations largely matched the observations in fungicide-exposed monocultures (Fig. 1a). Accordingly, fungicide exposure does not seem to affect biomass-specific functional performances. However, three out of these 16 comparisons indicated significant differences (Fig. 1a): Two cases are likely the result of fungicide-altered DNA concentrations (as discussed above) over-estimating leaf decomposition by *N. lugdunensis* (Baudy et al., 2020). Additionally, at a fungicide sum concentration of 500 μg/L, *T. marchalianum* decomposed more leaf mass than predicted (Fig. 1a). As this effect seems independent of fungicide mixture concentrations and could not be explained by enzyme activity or other variables (Fig. 1b; Fig. 2d), this observation may be the result of chance.

3.2. Responses of mixed cultures

Irrespective of the fungicide concentration, leaf mass loss, fungal biomass production and enzyme activity profiles in binary cultures and the quaternary culture show similar patterns as observed in the respective most productive and tolerant species in monoculture (Fig. 1; Fig 2). Accordingly and as hypothesized, functions are maintained in mixed cultures up to high fungicide concentrations (Pimentão et al., 2020; Zubrod et al., 2015a). For both functional variables, predictions significantly deviating from observations mainly concerned mixed AH cultures containing *N. lugdunensis* (6 out of 70 comparisons; Fig. 1), which is discussed above. Again, the few significant deviations concerning other mixed cultures (i.e., 3) are likely not the result of fungicide impacts, as these responses were not concentration-dependent and could not be explained by other variables (Fig. 1b; Fig. 2). Accordingly, and as observed in monocultures, fungicide exposure did not affect biomass-specific functional performances in mixed cultures.

In binary cultures, fungicide exposure resulted in a similar response pattern of individual species' DNA concentrations as observed in monocultures (Fig. 3). In the quaternary culture, however, exposure to already the lowest fungicide sum concentration (i.e., 5 μg/L) significantly reduced DNA concentrations of *A. acuminata* and *H. stellata* by 60% and 50%, respectively (Fig. 3a and b). This may be explained by an intensified resource competition in the quaternary culture further increasing sensitivity to fungicides in sensitive species (i.e., synergistic effects of multiple stress factors; Steinberg, 2012). While trade-offs between somatic (mycelial) growth and (asexual) reproduction cannot be ruled out (i.e., increased conidial production at the expense of mycelial
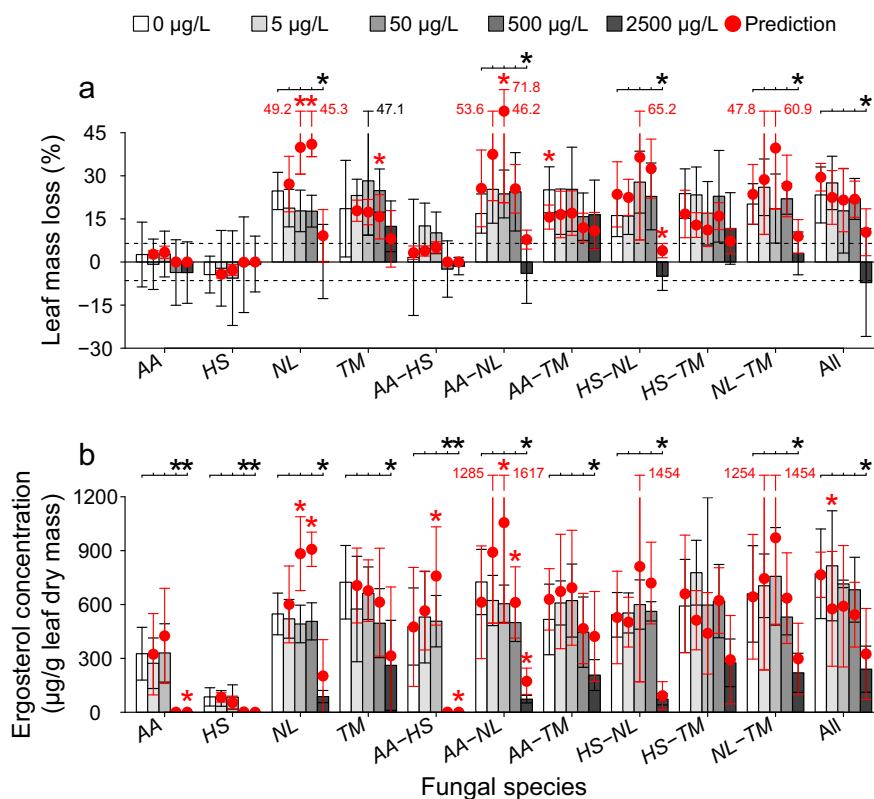
Fig. 1. Mean leaf mass loss (a) and ergosterol concentrations (b; with 95%-confidence intervals, respectively) in fungal species treatments exposed to fungicide sum concentrations of 0, 5, 50, 500, and 2500 μg/L (gray-scale bars) and mean predictions (red points with 95%-confidence intervals) based on biomass-specific leaf mass loss or ergosterol concentrations. Solid and dashed horizontal lines indicate the mean and 95%-confidence interval, respectively, of the sterile control (a). Black asterisks indicate significant differences between fungicide treatments and fungicide-free controls within fungal species treatments (Dunnett's test; $p < 0.05$; $n = 5$). Red asterisks indicate significant differences between observed and predicted variables (paired t-test; $p < 0.05$; $n = 5$). Note that predictions for each species are based on biomass-specific leaf mass loss and ergosterol concentrations in fungicide-free monocultures (therefore, mean predictions equal mean observations in these treatments; *AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, and *TM*: *T. marchalianum*). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

growth on the leaf substrate), it has been shown previously that fungicide exposure overall counteracts conidial production in AHs (Bundschuh et al., 2011; Dimitrov et al., 2014; Zubrod et al., 2015). Nonetheless and despite the low biomass of *A. acuminata* and *H. stellata* in the quaternary culture (compared to *N. lugdunensis* and *T. marchalianum*; Fig. 3), the present study confirms pronounced fungicide effects on community composition at environmentally relevant concentrations up to 50 μg/L.

This shift in community composition is accompanied by alterations in species interactions during leaf colonization. Net diversity effects, which are positively correlated with genetic divergence of the communities (i.e., synergistic interactions increase with genetic distance; Fig. S1; cf. (Baudy et al., accepted)), remained largely unaffected in binary cultures (Fig. 4). Yet, in the quaternary culture, net diversity effects were (partially significantly) reduced from 3% in the fungicide-free control to −32% and −38% at fungicide sum concentrations of 5 and 50 μg/L, respectively (Fig. 4g). These reductions are driven by significant alterations of trait-independent complementarity effects from positive, in the fungicide-free control (i.e., 35%), to negative, at fungicide sum concentrations of 5 and 50 μg/L (−7% and −32%, respectively; Fig. 4g). Accordingly and as

hypothesized, fungicide stress apparently induced a shift from complementary towards competitive interactions at the mutual expense of the community (Fox, 2005). Although in binary cultures net diversity effects remained unaffected, fungicide exposure lead to significant alterations of dominance between sensitive and tolerant species (Fig. 4c and d) as well as between the two tolerant species (Fig. 4f). The dominance of *T. marchalianum* over *A. acuminata* was slightly reduced from 31% in the control to 26% and 19% at fungicide sum concentrations of 5 and 50 μg/L, respectively (Fig. 4c). In contrast, the dominance of *N. lugdunensis* over *H. stellata* was substantially increased from 3% in the control to 9% and 43% at fungicide sum concentrations of 5 and 50 μg/L, respectively (Fig. 4d). In the binary culture comprising of the two most tolerant species, the dominance of *N. lugdunensis* over *T. marchalianum* seems to be maintained up to a fungicide sum concentration of 500 μg/L (−32% to −25%; Fig. 4f), retrospectively characterizing *N. lugdunensis* under environmentally relevant conditions as the most competitive species. As this relationship is reversed at a fungicide sum concentration of 2500 μg/L (31%; Fig. 4f), *T. marchalianum* seems to be the most tolerant species to fungicide stress (cf. Zubrod et al., 2015a).
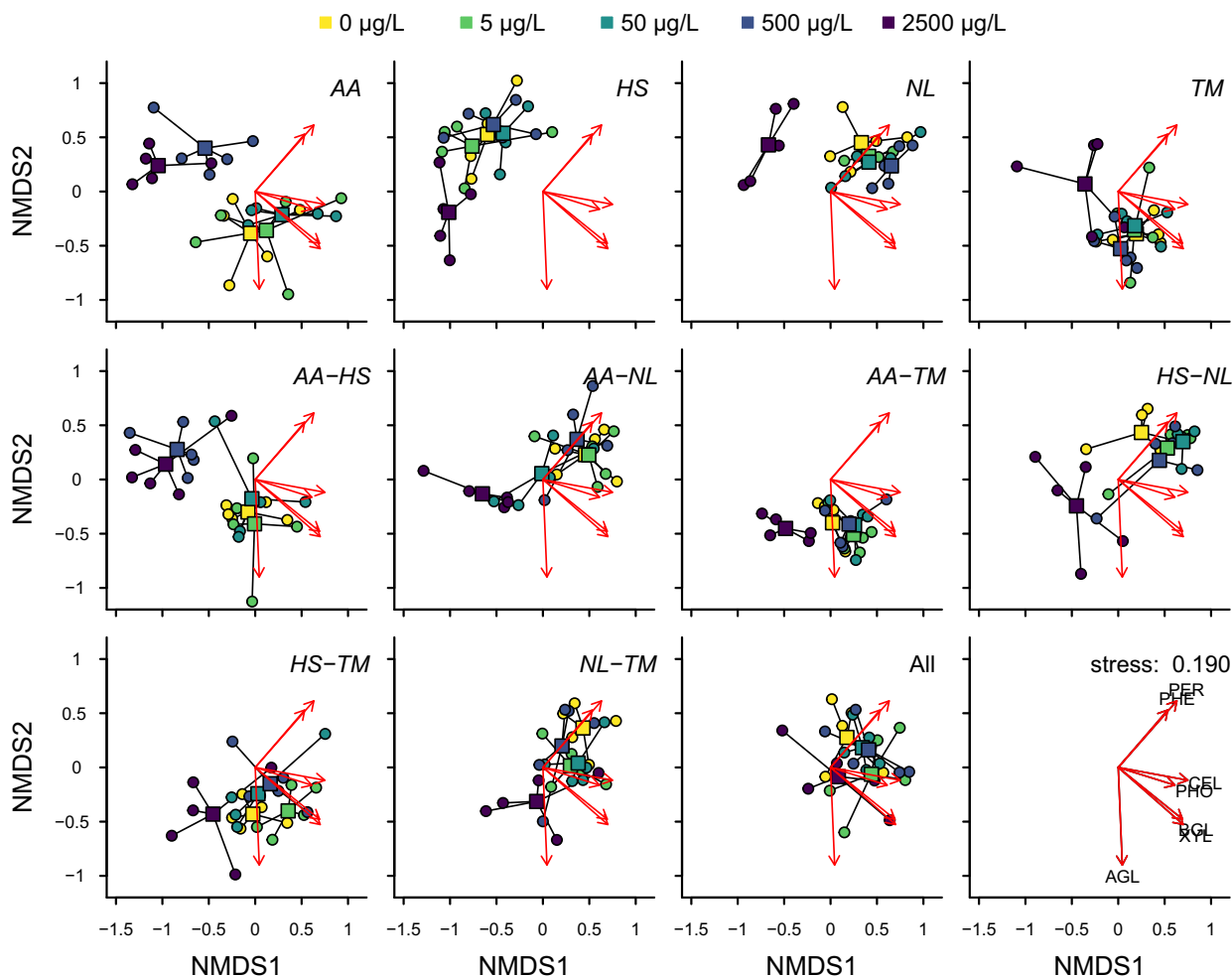
Fig. 2. Non-metric multidimensional scaling (NMDS) plots for enzyme activity profiles using Euclidean distance. Data shown in panels (a–l) originate from a common distance matrix. The panels show enzyme profiles for monocultures and mixed cultures exposed to fungicide sum concentrations of 0, 5, 50, 500, and 2500 μg/L. Group centroids and replicates are displayed as squares and circles, respectively. Red arrows display enzyme activities. The provided stress value indicates a reasonable fit (i.e., <0.2; Clarke, 1993). For the impact of the treatment factors on enzyme activity profiles, see Table S3 (*AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, *TM*: *T. marchalianum*, PHO: phosphatase, AGL: α-1,4-glucosidase, BGL: β-1,4-glucosidase, CEL: cellobiohydrolase, XYL: β-1,4-xylosidaseperoxidase, PER: peroxidase, and PHE: phenol oxidase). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. Implications for ecosystem functioning in streams

The results of this microcosm study indicate that AH-mediated leaf litter decomposition may not be affected by environmental fungicide exposures (Fig. 1a). As already low fungicide concentrations can have pronounced effects on community composition (Fig. 3) and diversity (Fernández et al., 2015), the maintenance of the decomposition process (in quantitative terms) seems to be safeguarded by highly tolerant species such a *N. lugdunensis* and *T. marchalianum* (Fig. 1), which together cover all of the investigated hydrolytic and oxidative enzymes involved in leaf degradation (Fig. 2c and d). Yet, the universality of these findings needs to be further validated, which may be pursued in future experiments using the tools applied in this study in combination with higher-tier test systems (e.g., indoor or outdoor stream mesocosms), involving

different AH species, higher fungal diversity, leaf substrates of varying toughness and fluctuating test conditions.

Analogous to leaf litter decomposition, it could be concluded that AH-mediated leaf conditioning in streams remains unaffected by environmental fungicide exposures, when considering solely total fungal biomass (i.e., ergosterol concentrations) as a surrogate variable for this process (Fig. 1b; Foucreau et al., 2013). However, the leaves' nutritional quality for consumers depends on fungal community composition (Danger et al., 2016). Assessment of community composition in the quaternary culture under fungicide exposure reveals a substantial decline of *A. acuminata* (Fig. 3a), an AH species known to be preferably consumed by stream invertebrates (Arsuffi and Suberkropp, 1989). AH species rather rejected by these consumers, on the contrary, persisted in this community, that is *N. lugdunensis* and *T. marchalianum* (Arsuffi and
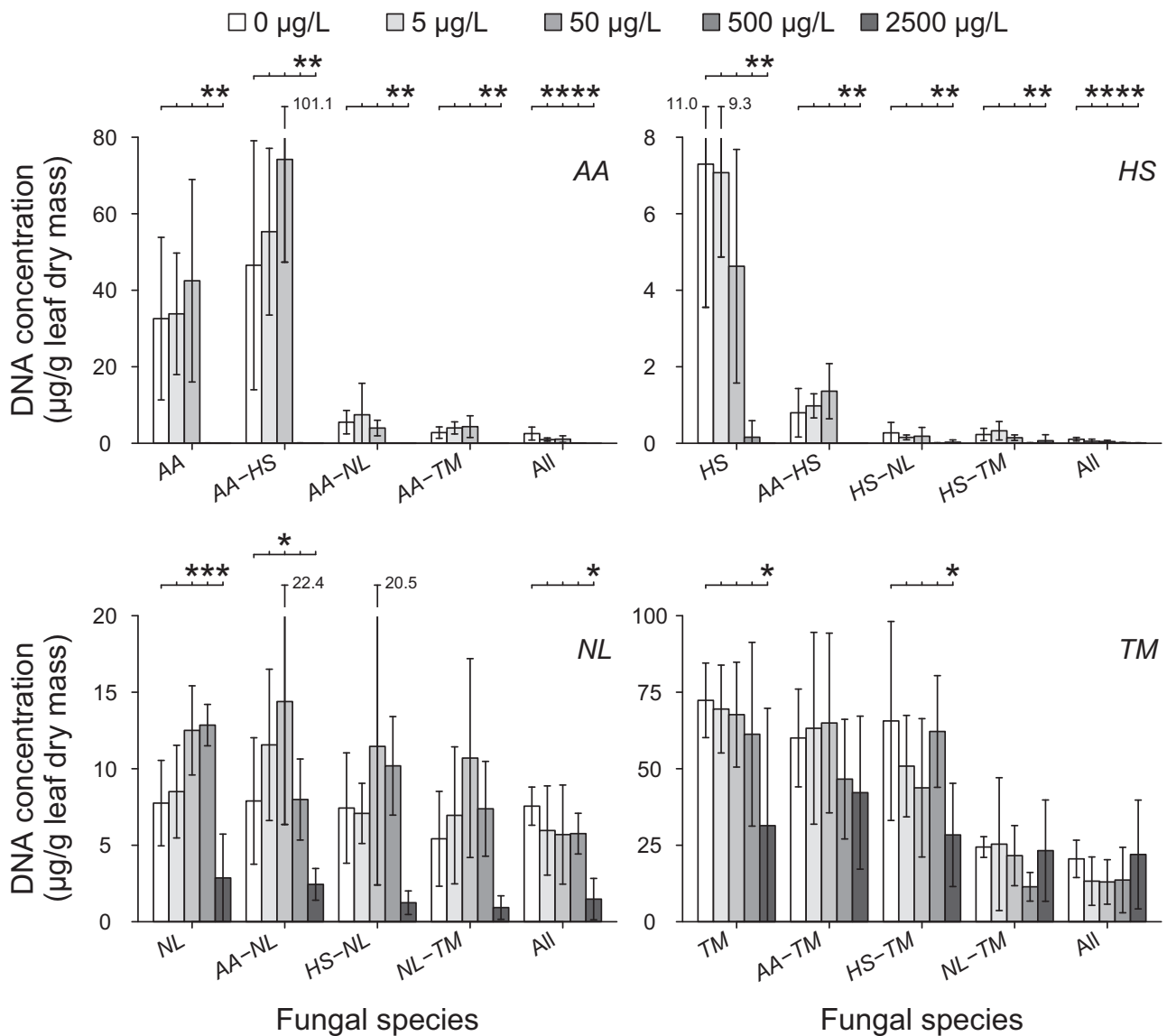
Fig. 3. Mean DNA concentrations (with 95%-confidence intervals) of *A. acuminata*, *H. stellata*, *N. lugdunensis*, and *T. marchalianum* in fungal species treatments exposed to fungicide sum concentrations of 0, 5, 50, 500, and 2500 μg/L (gray-scale bars). Asterisks indicate significant differences between fungicide treatments and fungicide-free controls (Dunnett's test; $p < 0.05$; $n = 5$; *AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, and *TM*: *T. marchalianum*).

Suberkropp, 1989; Rong et al., 1995). Moreover, these fungicide-induced alterations of AH community composition reflect those observed in earlier studies, demonstrating the above-discussed feeding preferences in an invertebrate leaf consumer (Bundschuh et al., 2011; Zubrod et al., 2015a). While mechanisms behind this selective feeding are not fully understood, a possible explanation might be fungal interspecific differences in the composition of macronutrients such as amino acids or essential fatty acids (as reviewed by Danger et al., 2016). Accordingly, chemical stress-induced alterations of AH community composition may affect the physiological fitness of consumers if alternative food sources are lacking (cf. Konschak et al., 2019, 2020).

4. Conclusion

Leaf mass loss and total fungal biomass production are, amongst others, the most widely used variables to assess the functional integrity of detritus-based stream ecosystems (Colas et al., 2019; Gessner and Chauvet, 2002; Graça et al., 2005). Although the stressors applied in this study (i.e., fungicides) specifically target organisms playing key functional roles in these ecosystems (i.e., fungi), no adverse functional effects were indicated at environmentally relevant concentrations. The presence of highly tolerant species combined with functional redundancy (with respect to these variables), however, masks alterations of AH community
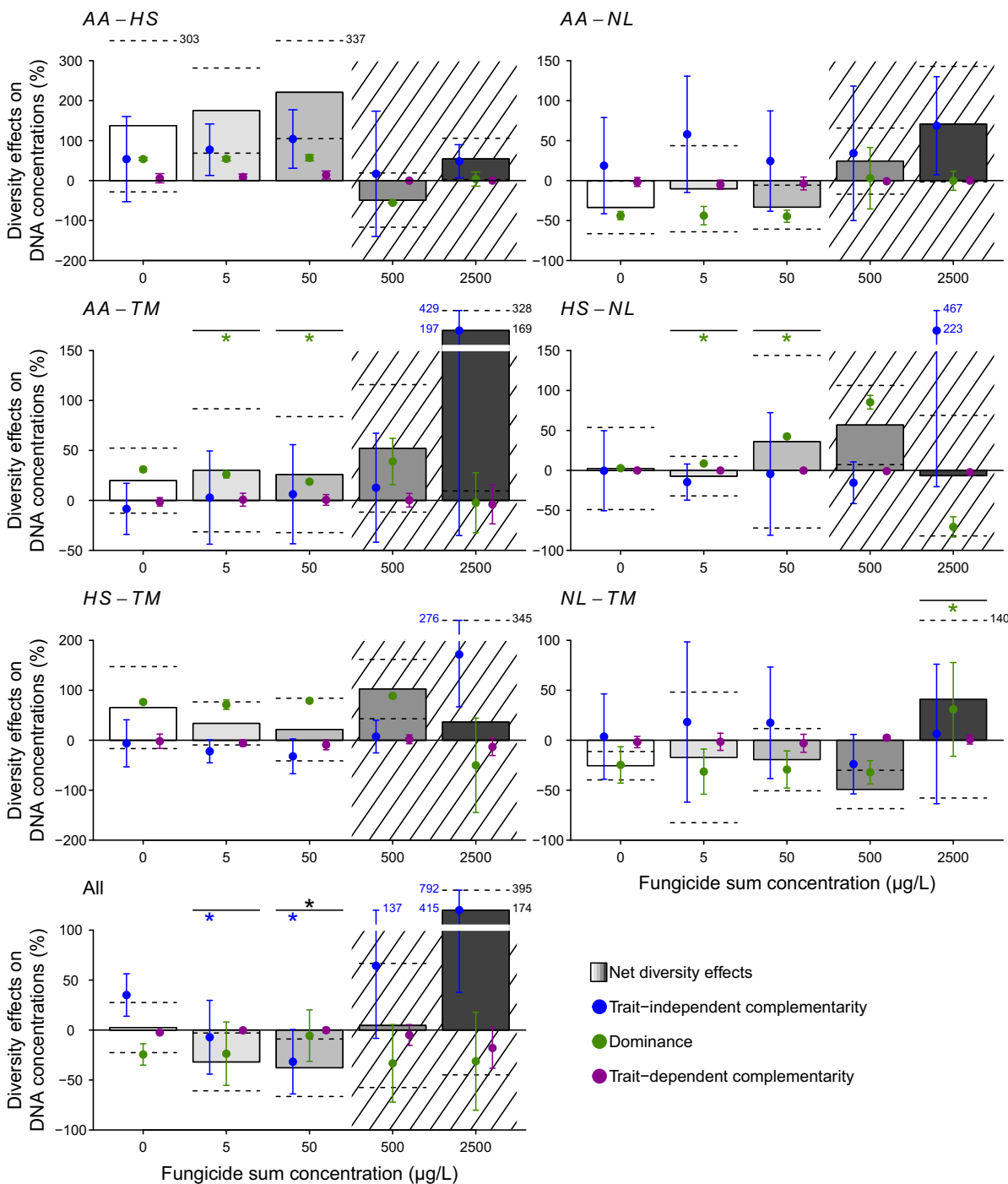
Fig. 4. Mean diversity effects on DNA concentrations (with 95%-confidence intervals) of mixed cultures exposed to fungicide sum concentrations of 0, 5, 50, 500, and 2500 μg/L. Net diversity effects (gray-scale bars) are composed of the sum of trait-independent complementarity (blue points), dominance (green points), and trait-dependent complementarity effects (purple points; for an explanation of these effects see section *Diversity effects on leaf colonization*). Black and colored asterisks indicate significant differences in net diversity effects or component interaction effects, respectively, between fungicide treatments and fungicide-free controls (Dunnett's test; $p < 0.05$; $n = 5$). Treatments plotted within shaded areas contain species, apparently incapable of surviving under the applied fungicide concentration, as no ergosterol could be detected in the respective monoculture treatment (Fig. 1b). Thus, these treatments likely do not reflect true interactions and are therefore excluded from statistical analysis (*AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, and *TM*: *T. marchalianum*). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

composition. Although previous studies provide a strong indication of a close link between AH community composition and leaf consumers' physiological fitness (Konschak et al., 2019, 2020), the

mechanistic basis of this relationship is not well understood and requires further research. Accordingly, filling this knowledge gap may foster the assessment of potential (fungicide) stress-induced

cascading effects on detritus-based stream food webs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117234.

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