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As above, so below? Effects of fungicides on microbial organic matter decomposition are stronger in the hyporheic than in the benthic zone

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Abstract

Microbial organic matter decomposition is a critical ecosystem function, which can be negatively affected by chemicals. Although the majority of organic matter is stored in sediments, the impact of chemicals has exclusively been studied in benthic systems. To address this knowledge gap, we assessed the impact of a fungicide mixture at three concentrations on the decomposition of black alder leaves in the benthic and hyporheic zone. We targeted two sediment treatments characterized by fine and coarse grain sizes (1–2 vs. 2–4 mm). Besides microbial communities' functioning (i.e., decomposition), we determined their structure through microbial biomass estimates and community composition. In absence of fungicides, leaf decomposition, microbial biomass estimates and fungal sporulation were lower in the hyporheic zone, while the importance of bacteria was elevated. Leaf decomposition was reduced (40%) under fungicide exposure in fine sediment with an effect size more than twice as high as in the benthic zone (15%). These differences are likely triggered by the lower hydraulic conductivity in the hyporheic zone influencing microbial dispersal as well as oxygen and nutrient fluxes. Since insights from the benthic zone are not easily transferable, these results indicate that the hyporheic zone requires a higher recognition with regard to ecotoxicological effects on organic matter decomposition.

Introduction

Allochthonous organic matter, such as leaf litter, is a major carbon and energy source for stream ecosystems (Vannote et al. 1980; Webster and Meyer 1997; Tank et al. 2010). Leaf-associated fungi and bacteria drive the ecosystem process of leaf decomposition (Cummins and Klug 1979; Gessner et al. 1999). Despite the importance of bacteria, microbial decomposition is typically dominated by aquatic hyphomycetes (Hieber and Gessner 2002) – a polyphyletic group of mitosporic fungi (Webster 1992). In addition to their direct contribution to leaf decomposition through the enzymatic degradation of structural polysaccharides (Taylor and Chauvet 2014), aquatic hyphomycetes increase the nutritious value of leaves for higher trophic levels (Triska 1970; Kaushik and Hynes 1971; Bärlocher 1992). Thereby they contribute directly and indirectly to the decomposition of leaves and the production of fine particulate organic matter (FPOM; Danger et al. 2016), an important food source for collectors (Short and Maslin 1977; Wallace et al. 1977). See Table 1 for the list of abbreviations.

As aquatic ecosystems are subjected to various pressures including organic and inorganic chemical stressors (reviewed by Bernhardt et al. 2017), aquatic microorganisms and the ecosystem functions they provide may also be affected. Particularly, fungicides have the potential to affect decomposing microorganisms and consequently leaf litter decomposition (reviewed by Zubrod et al. 2019). Since fungicides tend to be moderately lipophilic and have a high potential to adsorb to organic carbon (Zubrod et al. 2019), they are frequently detected in the μg kg^{-1} range in freshwater sediments with largely unknown environmental impacts (Smalling et al. 2013). Furthermore, there is evidence that fungicides are still fairly mobile in pore water (Reilly et al. 2012) suggesting their bioavailability to microorganisms.

However, studies assessing the impact of chemicals, such as fungicides, on leaf decomposition have, to our knowledge,

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Table 1. List of abbreviations.

ANOVA	Analysis of variance
FPOM	Fine particulate organic matter
GC-FID	Gas chromatography - flame ionization detector
HPLC	High-performance liquid chromatography
NLFA	Neutral lipid fatty acids
NMDS	Nonmetric multidimensional scaling
PERMANOVA	Permutative multivariate analysis of variance
PLFA	Phospholipid fatty acids
qPCR	Quantitative real-time polymerase chain reaction

exclusively been performed in the benthic zone. Potential implications on leaf litter decomposition in the hyporheic zone, the transitional zone in sediments between surface and groundwater (Orghidan 1959), have largely been ignored. This lack of information is worrying as hyporheic zones store up to one order of magnitude higher masses of organic matter compared to the benthic zone (Cummins et al. 1983; Metzler and Smock 1990; Jones et al. 1997) by burying leaves after, for example, flooding and sediment movement (Metzler and Smock 1990; Naegeli et al. 1995). Consequently, a large fraction of organic matter in sediments is of terrestrial origin (Goñi et al. 2003). Given that terrestrial organic matter is mainly particulate (POM) and aquatic organic matter predominantly dissolved (DOM), their relative ratios can differ in and above the sediment with potential repercussions on decomposing microbes (i.e., DOM–POM exchange processes, cf. He 2016). As environmental characteristics (e.g., hydrological conductivity, temperature, redox conditions, or nutrients; Findlay 1995) of the hyporheic zone may deviate substantially from its benthic counterpart, colonization and activity of leaf-associated microorganisms may deviate as well (Gulis and Suberkropp 2003; Bergfur et al. 2007; Clapcott and Barmuta 2010). Moreover, interactions of those environmental variables and chemical stress have recently been shown for decomposing benthic microorganisms (Feckler et al. 2018). In addition, sediments have a strong adsorption capacity for organic contaminants and are regarded as pesticide sinks (Warren et al. 2003), which poses a distinct exposure scenario for microbial communities. Consequently, fungicide effects in the hyporheic zone might differ from the benthic zone.

With the aim to address this knowledge gap, we assessed structural (i.e., fungal biomass and sporulation as well as general microbial biomass and community composition using phospholipid fatty acids as proxy; Frostegård et al. 2011; Quideau et al. 2016) and functional (i.e., leaf decomposition) changes induced by 3 concentrations of a fungicide mixture (Table 2) on leaf-associated microbial communities in hyporheic zones. Fungicide effects were assessed in hyporheic zones characterized by fine and coarse grain size, respectively, assuming differences in hydrological conductivity. Moreover, effects observed in the hyporheic zone were directly compared to those in the benthic zone within the same replicate ultimately resulting in a 2×2×3-full-factorial design.

We hypothesized that both leaf burial (Herbst 1980; Metzler and Smock 1990; Cornut et al. 2010, but *see* Rounick and Winterbourn 1983; Piscart et al. 2011; Solagaistua et al. 2016) and fungicide exposure (Bundschuh et al. 2011) reduce fungal and general microbial biomass leading to an impairment in leaf decomposition. We also hypothesized that the effect patterns induced by fungicide exposure are comparable between benthic and hyporheic zones, with effects being, however, less pronounced in the latter. This hypothesis is based on the premise that fungi are more sensitive to fungicide mixtures (Zubrod et al. 2015*a*) and the relative importance of fungi for leaf decomposition in relation to bacteria is lower in the hyporheic zone. This is assumed since some bacteria are capable of switching to anaerobic respiration in case of oxygen deficiency (Schwoerbel and Brendelberger 2010), but fungi cannot. Moreover, we hypothesized a shift in the fungal community composition with both increasing fungicide concentration (Bundschuh et al. 2011) and between the hyporheic and the benthic zone.

Materials and methods

Preparation of microbial inoculum

The preparation of inoculum largely followed Zubrod et al. (2015*c*). Briefly, black alder (*Alnus glutinosa* [L.] Gaertn) leaves were collected near Landau, Germany (49°11′N, 8°05′E) at the beginning of November 2016 and stored at −20°C. Approximately 10 leaves were placed in ~100 mesh bags (0.5 mm mesh size). Half of these bags were attached to seven poles and conditioned for 21 d in the benthic zone of a pool stretch of the Rodenbach near Grünstadt, Germany (49°33′N, 8°02′E), while the other half was attached to the same poles for 28 d buried in the hyporheic zone 5–10 cm below the sediment–water interface. With the different conditioning periods, it was aimed to counterbalance the expected differences in colonization dynamics between zones (Cornut et al. 2010) aiming for a representative community for both zones. After field conditioning, all leaves were mixed and placed for another 3 weeks at 16 ± 1°C in continuously aerated nutrient medium (Dang et al. 2005) together with uncolonized alder leaves. A separate conditioning of hyporheic and benthic inocula was not considered since the same starting conditions in both zones are imperative to reliably address the hypotheses posted. New uncolonized leaves were added weekly to ensure substrate diversity and therefore microbial communities characteristic for variable decomposition stages (Gessner et al. 1993). The nutrient medium was changed weekly.

Experimental design

The experiment employed a 2×2×3-full-factorial design (Fig. 1) involving two experimental zones (above- and within-

Table 2. Products, producers, modes of action, and nominal concentrations of the fungicides.

Fungicide	Product	Producer	Mode of action (FRAC 2019)	$Log_e K_{OC}$ (Lewis et al. 2016)	Nominal concentration (μg L^{-1})
Azoxystrobin	Ortiva	Syngenta Agro	Inhibition of mitochondrial respiration	6.05	0; 6; 60
Carbendazim	Derosal	Bayer CropScience	Inhibition of mitosis and cell division	5.42	0; 6; 60
Cyprodinil	Chorus	Syngenta Agro	Inhibition of amino acid and protein synthesis	7.73	0; 6; 60
Quinoxyfen	Fortress 250	Dow AgroSciences	Perturbation of signal transduction	10.04	0; 6; 60
Tebuconazole	Folicur	Bayer CropScience	Inhibition of sterol biosynthesis	6.65	0; 6; 60
Mixture	All above	All above	All above		0; 30; 300

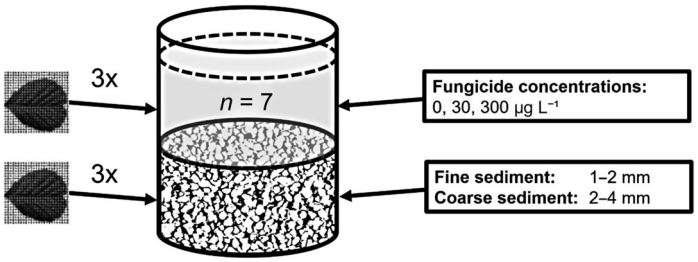

Fig. 1. Schematic representation of the experimental setup. After distributing microbial inoculum homogeneously in and above the sediment, three leaf bags were positioned both in the benthic and the hyporheic zone. Each of the six possible variable combinations (i.e., two sediment types and three fungicide sum concentrations) was replicated seven times, containing one benthic and one hyporheic sample (each with three pseudoreplicates). The medium was renewed weekly to ensure continuous fungicide and nutrient exposure. The experiment was run for 21 d.

sediment) crossed with two sediment grain sizes (fine and coarse) and three increasing concentrations of a fungicide mixture (control, low, and high). For the sake of simplicity, the zones above and within the sediment will be referred to as "benthic" and "hyporheic," respectively, although they may not fully satisfy the definition of their natural equivalent. The two sediment grain sizes fine and coarse were realized by using sand/gravel with a size of 1–2 and 2–4 mm in diameter (both: Siligran EN 12620, Euroquarz GmbH), respectively. These grain size classes are common in woodland streams (Burrows et al. 2014; Cornut et al. 2014) and are thus considered to be environmentally relevant. The three concentrations of the fungicide mixture included a fungicide–free control, sum concentrations of 30 μg L^{-1} (low) reflecting environmentally relevant concentrations (Bereswill et al. 2012) and 300 μg L^{-1} (high) to test for concentration dependency in the observed effects. The applied fungicide mixture contains compounds with different modes of action targeting distinct cell processes (Table 2) and being commonly found in freshwater systems (Orlinskiy et al. 2015; Landesamt für Umwelt 2016). Moreover, the same mixture was used in earlier studies, in which effects in benthic microbial leaf litter decomposition were reported (Zubrod et al. 2015*b*; Feckler et al. 2018), allowing a direct comparison among studies. To simulate agricultural practices, commercially available fungicide formulations were used containing the active ingredient as well as formulants, which were solved in nutrient medium (Dang et al. 2005) before being used in the experiment. Stock solutions were diluted appropriately to achieve the nominal concentrations (Table 2). To verify nominal concentrations, water samples were taken 7 days after test initiation before and after the renewal of the medium from two replicates per treatment, zone and grain size ($n = 48$) and stored frozen until chemical analysis. The samples were analyzed using a liquid chromatography high-resolution mass-spectrometry Orbitrap system (Thermo Fisher Scientific) with matrix-matched standards ranging from 0.1 to 150 μg L^{-1} (for details, *see* Fernández et al. 2016). Despite discrepancies between nominal and measured concentrations (Table S2), fungicide concentrations by "low" and "high" differed one order of magnitude, which allows keeping the hypothesis unchanged. In 3 of 16 measured control replicates, fungicides were detected in the sub-μg L^{-1} range suggesting random contamination at ecotoxicologically irrelevant levels.

The experiment was performed over 21 d in 5-liter aquaria ($n = 7$), which were filled with 2.5-liter sediment and 3-liter continuously aerated (via air stones) nutrient medium (Dang et al. 2005), containing respective fungicide concentrations. The test was conducted in complete darkness to reduce complexity potentially introduced by algal priming (Halvorson et al. 2019). To reduce the availability of sorption sites in the sediment, the replicates were dosed 3 d before the actual start of the experiment. At test initiation, the nutrient medium containing the respective fungicide concentration was renewed – a process that was repeated in weekly intervals.

At the start of the experiment, 10 ± 0.1 g wet weight of microbial inoculum (*see* the "Preparation of microbial inoculum" section) was crushed manually (leaf fragment area < 6.25 cm^2) and mixed homogeneously in the hyporheic zone. The same amount was placed in the benthic zone. This procedure served the colonization of unconditioned test leaves in the respective zones by leaf-associated microorganisms. For each replicate, a total of 0.54 ± 0.1 g (dry weight) unconditioned test leaves were cut in squares, placed in three mesh bags (5×4 cm, mesh size: 0.5 mm; Navel et al. 2013) and deployed in each zone (i.e., three leaf bags per zone). Although drying of leaves increases leaching of soluble compounds and can alter colonization (Bärlocher 1997), it does not affect comparisons among treatments. These three bags (i.e., pseudotriplicates) accounted for spatial heterogeneity in both zones (Brunke and Gonser 1997) and were treated as one statistical replicate (*see* the "Calculations and statistics" section). The bags used in the hyporheic zone were placed approximately 5 cm below the sediment–water interface, which is an ecologically relevant depth (Cornut et al. 2012). After 21 d, leaf bags were retrieved and adhering sediment particles were gently hosed down with water. Subsequently, nine leaf discs (diameter: 10 mm) were punched per replicate (three per pseudotriplicate) and preserved for sporulation analysis (*see* the "Microbial endpoints" section) as well as for the estimation of leaf disc dry mass. The remaining leaf material was freeze-dried (Alpha 1-4 LSCbasic, Christ), weighed to the nearest 0.01 mg for the determination of leaf mass loss (*see* the "Calculations and statistics" section) and stored at −20°C until further analyses (ergosterol as well as neutral and phospholipid fatty acids). To correct for leaching, a separate set of leaf bags was placed in three control aquaria for 3 d and processed likewise.

Microbial endpoints

Ergosterol

Ergosterol, a component of the cell membrane of true fungi and a proxy for fungal biomass (Newell et al. 1988), was quantified according to the method described by Gessner (2005). Briefly, freeze-dried leaves were lipid extracted in KOH/methanol. Ergosterol was purified using solid-phase extraction (Sep-Pak Vac RC 500 mg tC18 cartridges, Waters Corp.) and quantified via high-performance liquid chromatography (HPLC, 1200 Series, Agilent Technologies) with a LiChrospher®100 RP 18–5 µm column (250.0 mm × 4.6 µm, particle size 5 µm, CS-Chromatographie Service) at 282 nm. An external calibration curve enabled quantification (Ergosterol ≥ 95.0% HPLC, Sigma-Aldrich). Ergosterol is expressed as µg g^{-1} dry leaf mass.

Sporulation

Fungal sporulation provides insight into the dispersal and composition of fungal communities (Bärlocher 2004) and was analyzed as per Pascoal and Cassio (2004). Briefly, three leaf discs were placed on an orbital shaker in 10 mL of sterile, deionized water at 120 rpm in the dark for 48 h. To prevent spores from attaching to the leaves, 25 µL of 0.5% Tween® 80 was subsequently added as surfactant. For analyses, 5 mL aliquots of each sample were filtered (diameter: 47 mm, pore size: 0.45 µm, Millipore) and stained with lactophenol cotton blue. Conidia were identified under a microscope (Di-Li 2026-P, Distelkamp Electronic) with up to 400-fold magnification according to several identification keys (Gulis et al. 2005). The number of spores was normalized to the dry leaf mass.

Fatty acid analysis

Fatty acids were analyzed as described by Konschak et al. (2020). Briefly, lipids were extracted from freeze-dried leaves with a chloroform/methanol/Milli-Q-water mixture (1:2:0.8, V/V/V, modified from Bligh and Dyer 1959). Neutral lipid fatty acids (NLFA, i.e., leaves' energy reserves) and phospholipid lipid fatty acids (PLFA, i.e., proxy for active microbial biomass; Bååth 2003) were separated and purified using solid-phase extraction (Chromabond® Easy polypropylene columns, Macherey-Nagel). Samples were concentrated under a nitrogen-atmosphere at 40°C using a dry bath heater (EC-1V-130, VLM). Transesterification (i.e., methylation; Butte 1983) was conducted with trimethylsulphonium hydroxide (TMSH, 0.25 M in methanol, for gas chromatography derivatization, Sigma-Aldrich).

Samples were measured by gas chromatography with a flame ionization detector (GC-FID, Varian CP-3800, Agilent) equipped with a capillary column (J&W DB-225, length: ~ 30 m, diameter: 0.25 mm, film thickness: 0.25 µm, Agilent) using nitrogen as carrier gas. Measurements were calibrated with four 10-point external standard curves (Supelco 37 Component FAME Mix, Sigma-Aldrich; Mixture BR 3, Larodan) and corrected for recovery by adding deuterium-marked internal standards (for NLFAs: Tristearin-D105; for PLFAs: 1,2-Distearoyl-D70-3-sn-Glycerophosphatidylcholine; both Larodan) prior to the extraction from leaves. External standards were measured throughout the analysis to correct for component-specific changes in peak areas across all runs if the sensitivity (i.e., slope of peak area vs. run number) was significant and the determination of the linear regression was acceptable (i.e., $R^2 > 0.8$). The resulting sample concentrations were normalized to the leaves' dry weight. Since the PLFAs "16:0" and "18:3 ω-3" acids are mostly of plant origin (Bundschuh et al. 2021), they were excluded from the analysis.

Calculations and statistics

Relative microbial leaf mass loss (ξ) in percent was calculated as

$$\xi = \left(1 - \frac{(3 \cdot m_{\text{discs}} + m_{\text{rest}})}{m_{\text{start}}} + f_{\text{leaching}}\right) \cdot 100\% \quad (1)$$

where m_{discs} is the dry mass of three leaf discs punched out for mass calculation multiplied times three to correct for the three

triplets removed from the remaining leaves (*see* the "Experimental design" section), m_{rest} is the dry mass of the remaining leaves after leaf discs had been punched, m_{start} is the dry mass of a replicate (one leaf bag triplet) before the experiment and $f_{leaching}$ is the relative mass loss due to physical leaching calculated as

$$f_{\text{leaching}} = 1 - \frac{m_{\text{end}}}{m_{\text{start}}} \quad (2)$$

where m_{end} is the dry mass of one leaf bag triplet after 3 d of leaching. The bacterial–fungal ratio (BF_{ratio}), which is used to estimate the relative importance of bacteria and true fungi, was calculated as

$$\text{BF}_{\text{ratio}} = \frac{\beta_{\text{Bacteria}}}{\beta_{\text{Ergosterol}}} \quad (3)$$

where $\beta_{Bacteria}$ is the sum mass concentration of PLFAs inter alia from bacterial origin (Table S5; Willers et al. 2015) and $\beta_{Ergosterol}$ is the mass concentration of ergosterol (both per gram leaf dry mass). Ergosterol was preferred over PLFAs of fungal origin as fungal biomass proxy because of the low specificity of the latter (Frostegård et al. 2011). PLFAs from bacterial origin were preferred over bacterial counts since the latter leads to a higher variability due to bacteria clusters on the leaves' surface.

For every endpoint normality of the data was checked with quantile–quantile plots and variance homogeneity was checked using Levene's test. Effects of sediment grain size, the zone in which the leaf bags were located and the fungicide treatment on leaf mass loss, ergosterol concentration, total NLFAs, total PLFAs, and bacterial–fungal ratio were tested using a three-way ANOVA with the zone in which the sample is positioned (i.e., benthic and hyporheic) being nested within aquaria. Tukey test was used as post–hoc test to determine where statistically significant differences occurred among fungicide treatments (after two-way ANOVAs performed separately on the hyporheic and benthic data sets). For post–hoc comparisons between benthic and hyporheic zone (dependent) as well as fine and coarse sediments (independent) for a given fungicide treatment, Student's *t*-tests (paired and unpaired, respectively) with Benjamini–Hochberg *p*-value adjustment were performed.

Fungal community data, NLFAs, and PLFAs were double square-root transformed to reduce the weight of highly abundant species/fatty acids (Clarke and Warwick 2001) and species/fatty acids only present in one sample were excluded from the data to reduce arbitrary noise. Since the Bray–Curtis dissimilarity is undefined for samples without counts (Clarke et al. 2006) a "dummy species" with an abundance of 1 was added to the fungal community data. To test for significant differences among fungal communities and fatty acid fingerprints, permutative multivariate ANOVA (PERMANOVA; Anderson 2001; McArdle and Anderson 2001) was performed. For PERMANOVA, 999 permutations and a Bray–Curtis dissimilarity matrix were used. For testing each fungicide concentration against each other, *p*-values from pairwise comparisons using PERMANOVA were adjusted for multiple comparisons using the Benjamini–Hochberg method. However, since 80% of hyporheic sporulation samples did not contain any spores, hyporheic fungicide treatments were not statistically analyzed. Fungal community composition, NLFA and PLFA composition were visualized using nonmetric multidimensional scaling (NMDS) calculated with a Bray–Curtis dissimilarity matrix and a stress function ranging from 0 to 1 was used as a goodness of fit measure. All calculations, statistics and data visualizations were performed using the statistics software R (3.3.2, R Core Team 2019) with the add-on packages "vegan" (2.5-5, Oksanen et al. 2019), "car" (3.0-3, Fox and Weisberg 2019), "ggpubr" (0.2, Kassambara 2020), and "ggplot2" (3.1.1, Wickham 2016). The significance level was set at $\alpha < 0.05$ and used alongside effect sizes for the interpretation of results in order to take the criticism of exclusive null hypothesis significance testing (Newman 2008) into account.

Results and discussion

Comparison of the benthic and hyporheic zone

As hypothesized, leaf decomposition (pooled for both grain sizes) in the hyporheic zone was significantly lower than in the benthic zone in absence of fungicide stress (40%, $p < 0.001$; Fig. 2a). Although there was a 16% lower leaf decomposition in fine relative to coarse sediment, the general functional and structural pattern was not meaningfully different, justifying their combined discussion in the following (detailed statistical output of nested ANOVAs and unnested ANOVAs provided in Tables S1 and S2, respectively). The lower decomposition in the hyporheic zone went along with a 38% higher concentration of NLFAs (i.e., leaves' energy reserves, $p < 0.001$; Fig. 2c) and ultimately a different NLFA composition ($p < 0.001$; Fig. 4b). As NLFA concentrations tend to decrease over the course of decomposition (Torres-Ruiz and Wehr 2010), the observed difference in NLFAs shows an earlier stage of leaf decomposition in the hyporheic zone. This reduced decomposition is likely related to the 44% and 89% lower levels of microbial biomass (i.e., PLFA concentration) and especially fungal biomass (i.e., ergosterol concentration), respectively, in the hyporheic zone (Fig. 3a,c) suggesting a lower microbial activity (Mayack et al. 1989; Naamane et al. 1999; Cornut et al. 2010). These differences in ecosystem function and microbial community structure (i.e., species composition) are most likely explained by the low oxic conditions (Crenshaw et al. 2002; Medeiros et al. 2009) or a reduced colonization due to a hindered dispersal in the hyporheic zone (Cornut et al. 2010). Although the former was not explicitly assessed, it is a defensible assumption since oxygen is typically only present in several top millimeters of sediments

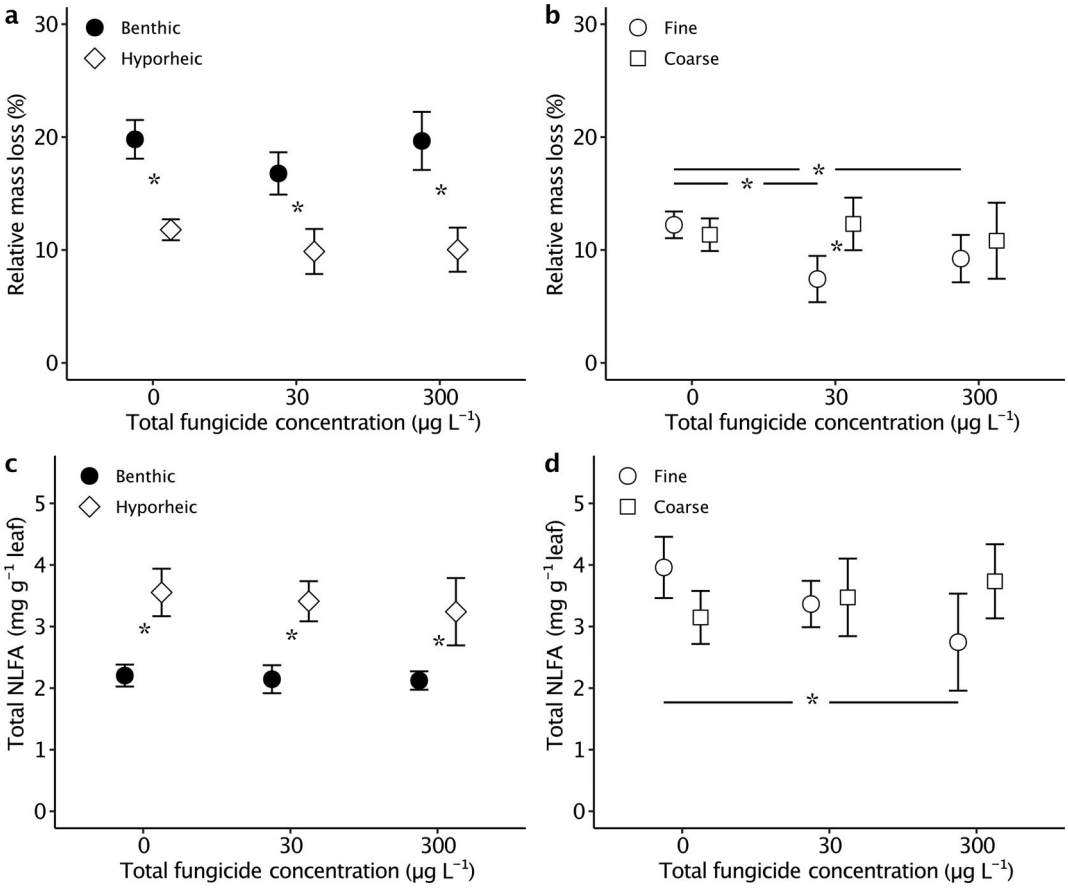

Fig. 2. Mean relative leaf mass loss (**a**, **b**) and mean NLFA content per gram leaf dry weight (**c**, **d**) with 95% confidence intervals. In (**a**) and (**c**), filled circles and open diamonds refer to benthic and hyporheic data (pooled for both grain sizes), respectively. In (**b**) and (**d**), open circles and squares represent fine and coarse sediment data, respectively. Solid lines with asterisks connect samples between which a significant fungicide effect was detected, while asterisks between symbols indicate a significant difference between the benthic and hyporheic zone or fine and coarse sediment, respectively, at a specific fungicide concentration.

(Mügler et al. 2012). Furthermore, fungi show in general – despite generally present adaptations (Hillmann et al. 2015) – a decreased biomass and decomposition efficiency under hypoxia (Medeiros et al. 2009).

Although both fungal and bacterial biomasses are reduced, the bacterial–fungal ratio was higher in the hyporheic zone (Fig. 3e; Table S1) suggesting a higher importance of bacteria for leaf decomposition in the hyporheic zone. This pattern was anticipated as reductions in fungal activity in response to oxygen deficiency are vast (Medeiros et al. 2009) and the biogeochemical processes conducted by bacteria (Storey et al. 1999) suggest their importance. Even so, the role of fungi in sediments is understudied but can be principal to the microbial network (Booth et al. 2019) and might depend on sediment type, flow characteristics and oxygen levels (cf. Martínez 2020). Nonetheless, the significant difference between the benthic and hyporheic zone regarding the general microbial (i.e., structure of PLFAs, $p < 0.001$; Fig. 4a) and fungal community structure ($p < 0.001$; Fig. 4c) supports the fundamental role of community structure for decomposition (Bier et al. 2015).

The differing structure of microbial communities in the hyporheic zone might be triggered by hindered dispersal. Although some studies state a ready dispersal of fungi in the hyporheic zone (Bärlocher and Murdoch 1989; Bärlocher et al. 2006, 2008), the reduction in sporulation by 99% (ANOVA, $p < 0.001$) in the hyporheic zone together with the low fungal biomass suggests a lower dispersal efficiency in our test system, which is likely triggered by the lack of a current and turbulences (Webster 1959; Bärlocher 1992). However, part of this low sporulation could be due to the presence of dormancy stages – which are ametabolic and therefore unlikely to contribute to leaf decomposition – that might have needed a longer period than 48 h to sporulate (cf. Mysyakina et al. 2016). As the decomposition efficiency of fungal communities tend to improve with species richness (Setälä and McLean 2004; Feckler and Bundschuh 2020), the reduction from a total of 14 species of aquatic hyphomycetes in the

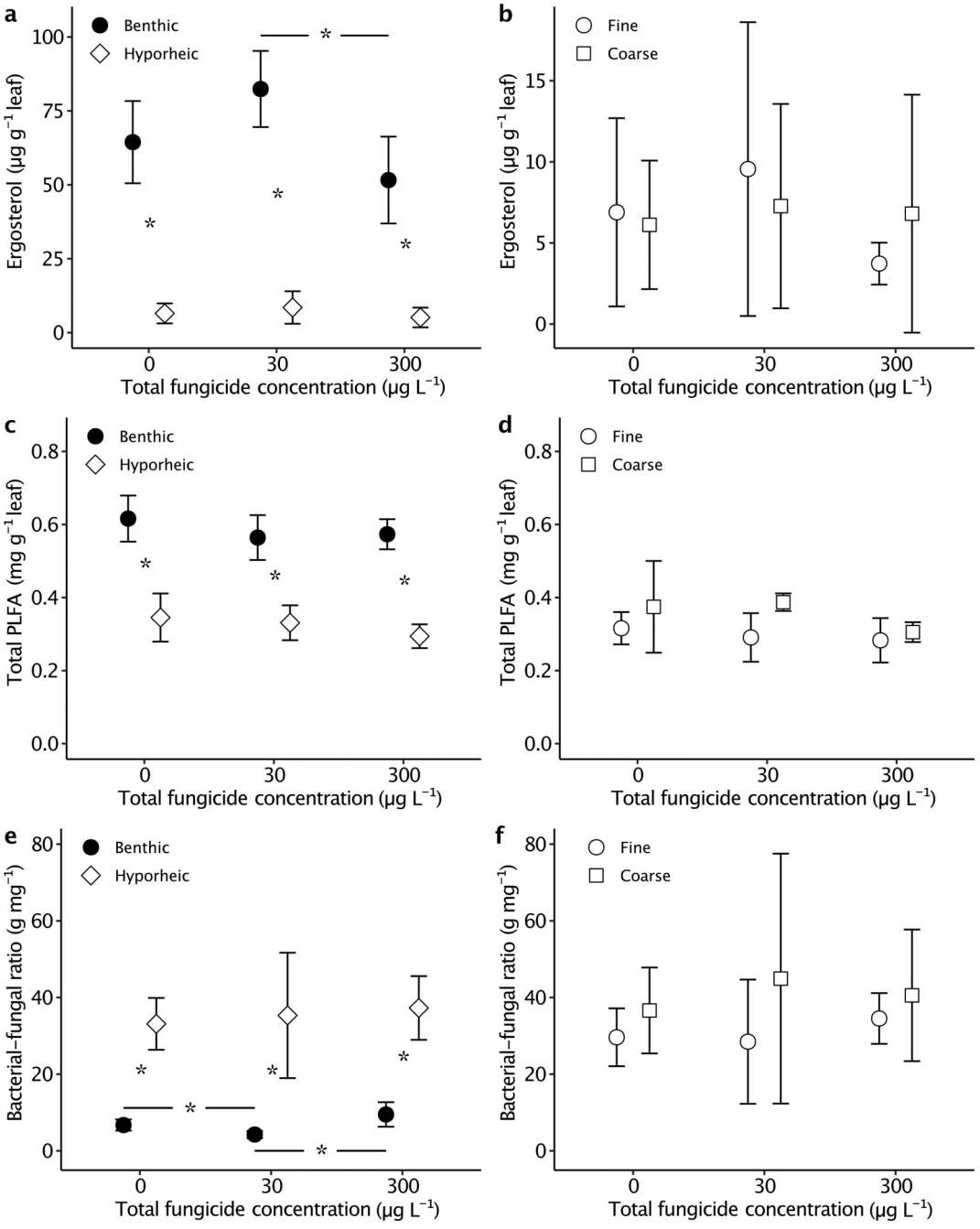

Fig. 3. Mean ergosterol content (**a**, **b**), PLFA content (**c**, **d**) and bacterial-fungal ratio (**e**, **f**) with 95% confidence intervals. In (**a**), (**c**), and (**e**) filled circles and open diamonds refer to benthic data and hyporheic data (pooled for both grain sizes), respectively. In (**b**), (**d**), and (**f**), open circles and squares represent fine and coarse sediment data, respectively. Solid lines with asterisks connect samples between which a significant fungicide effect was detected while asterisks between symbols indicate a significant difference between benthic and hyporheic zone and fine and coarse sediment, respectively, at a specific fungicide concentration.

benthic to six species in the hyporheic zone (Table S3), might serve as a supplemental explanation for the low decomposition rate. Besides the potentially increasing role of bacteria in the leaf decomposition in the hyporheic zone, other fungi than aquatic hyphomycetes, that cannot be detected by the methods (sporulation and ergosterol analyses) involved in this work, may have become more relevant for the measured ecosystem function. These species might include zoosporic fungi (i.e., Blastocladiomycota and Chytridiomycota) and stramenopiles, which are thought to be involved in leaf

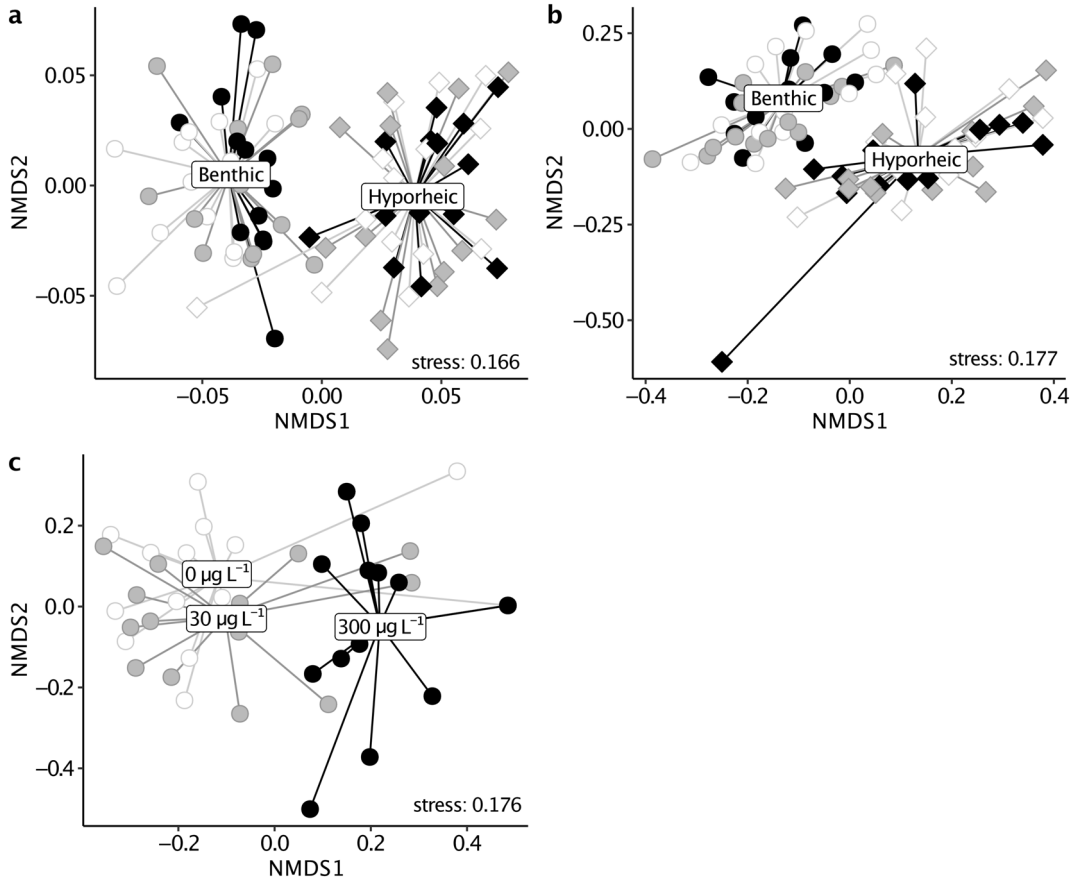

Fig. 4. NMDS ordination plots for benthic (circles) and hyporheic (diamonds) PLFA structure (**a**), NLFA structure (**b**) and benthic hyphomycete community structure (**c**) at 0 (white), 30 (gray), and 300 µg L^{-1} (black). Stress values indicate the "goodness-of-fit" of the NMDS with values below 0.2 indicating a reasonable fit (Clarke, 1993).

decomposition (Dix and Webster 1995; Gessner et al. 2007). Although their biomass is captured by PLFA measurements, group-specific assignments of fatty acids are uncertain, substrate-dependent (Frostegård et al. 2011) and thus not clear cut. In conclusion, the observed difference in microbial leaf decomposition between the benthic and hyporheic zone seems best explained by microbial biomass estimates with community structure supporting the interpretation.

Effects of fungicides in the benthic zone

Although several studies reported mostly negative effects of organic fungicides on benthic leaf decomposition at comparable concentrations (Bundschuh et al. 2011; Artigas et al. 2012; Zubrod et al. 2015*a*), leaf decomposition in this zone was nonsignificantly reduced by 15% at the low fungicide concentration, while the mass loss of the high fungicide treatment was not different to the fungicide-free control (Fig. 2a). Despite the importance of fungi for leaf decomposition, observed fungicide effects on fungal biomass accrual (i.e., ergosterol concentration) did not match with the observed trend for functioning (*see* also Feckler and Bundschuh 2020). At the low fungicide concentration ergosterol was nonsignificantly increased by 28%, while being nonsignificantly reduced by 20% at the high concentration when compared to the control (Fig. 3a). Using a similar test design to assess benthic fungicide effects, Zubrod et al. (2015*a*) also determined a higher ergosterol concentration at relatively low concentrations of tebuconazole and quinoxyfen. This might be explained by the ability of fungi to mineralize organic xenobiotics (Cabras et al. 2000; Krauss et al. 2011) and use them as an energy resource. Since also the general microbial biomass (i.e., PLFA concentration) was only nonsignificantly reduced by 8% at the low concentration (Table S2), the observed trend in functional response to fungicide exposures (i.e., leaf mass loss) might be explained by a shift in the fungal community structure.

Indeed, the fungal community composition was significantly altered by fungicide exposure ($p < 0.001$; Fig. 4c). Between the control and high fungicide concentration a significant difference occurred ($p = 0.003$), while between control and low concentration a trend was detected ($p = 0.075$). Although not being directly assessed in the present study,

these shifts may have affected the communities' leaf decomposition efficiency as fungal species can be different in their decomposition rates (Duarte et al. 2006; Baudy et al. 2021). Across all fungicide treatments *Tetracladium marchalianum*, *Neonectria lugdunensis*, and *Clavatospora longibrachiata* were the three most abundant species contributing at least 54% to total sporulation (Table S3). The relative proportion of *T. marchalianum* and *N. lugdunensis* to total sporulation increased (ANOVA, $p = 0.001$) and the relative proportion of *C. longibrachiata* in the total sporulation decreased with increasing fungicide concentration (ANOVA, $p < 0.001$). The insensibility and sensibility, respectively, of those species to fungicides have also been backed up by field studies assessing the tolerance of fungal communities to pesticides (Feckler et al. 2018). The dominance of tolerant species (e.g., *T. marchalianum*, for which higher breakdown rates have been reported; Andrade et al. 2016) could counteract fungicide effects on functioning (Baudy et al. 2021). Conversely, the fact that the lowest leaf mass loss was observed at the highest absolute spore number of *T. marchalianum* underlines that community functions cannot easily be deduced from performances of monocultures (Duarte et al. 2006) and are a result of complex interspecific relationships and functional redundancy (Pascoal and Marvanová 2005). In this context, a promising method to better fathom bacterial and fungal communities and their functioning is the use of group-specific quantitative real-time polymerase chain reaction (qPCR; Feckler et al. 2017). This is particularly useful for fungi since it is not reliant on a reproductive life phase (i.e., spores; Nikolcheva et al. 2003), a biomass proxy is a better predictor of decomposition than sporulation (Duarte et al. 2006) and it can quantify metabolically active fungal biomass with species-specific precision (Raidl et al. 2005; Baudy et al. 2019).

Effects of fungicides in the hyporheic zone

An effect of fungicides on leaf decomposition in the hyporheic zone was only observed in the fine sediment ($p = 0.006$; Fig. 2b) with a statistically significant reduction by 39% at the low and 25% at the high concentration when compared to the control, and thus a twofold higher effect size compared to the benthic zone. However, this effect in functioning cannot be explained by any structural endpoint used in the present study (i.e., ergosterol, sporulation and PLFAs; Table S2). Still, ergosterol concentration in the fine sediment showed with an increase by 41% for the low concentration and a decrease by 49% for the high concentration (compared to the control) an ecologically relevant response, which was, however, not statistically significant. Furthermore, due to the insubstantial sporulation in the hyporheic zone (*see* the "Comparison of the benthic and hyporheic zone" section), changes in fungal community composition, potentially underlying the observed functional responses, could not be determined. Moreover, the observed effects of fungicides on leaf mass loss might have also been caused by changes in the decomposition efficiency of the microbial community. A potential sign that could support this hypothesis is the negative monotonic fungicide effect on NLFA concentration of leaves buried in the fine sediment ($p = 0.032$; Fig. 2d) that may show a different usage of the leaves' components by the microbial community (compared to the communities in the coarse sediment). This might be indicated by the statistical interaction of fungicide and grain size effects on the assessed NLFA structure ($p = 0.0107$) in the hyporheic zone. Following this, microbial communities could preferably metabolize components with different catabolic rates in the assessed fungicide scenarios. Utilizing proteomic methods (Schneider et al. 2010) could help understanding potential changes in metabolic activity, which could elucidate the observed effect in functioning and address the suggested hypothesis.

Additionally to the leaves' NLFA structure, for both leaf decomposition and leaves' NLFA concentration there were interactions between fungicide and grain size effects (Table S2), indicating a sediment-substrate-dependency of fungicide effects. These interactions may be triggered by an altered oxygen and nutrient flux across the sediment–water interface (Babich and Stotzky 1983; Gadd et al. 2001) as grain size is regarded as the most important parameter affecting the hydraulic conductivity (Sperry and Peirce 1995). Consequently, it may also be assumed that differences in pore water fungicide concentrations between the two grain sizes could explain the distinct fungicide effects. However, such differences could not be identified from the water fungicide concentrations, making this assumption unlikely (Table S4). More likely, sorption of organic fungicides (such as used in this study) to leaves or leaf-derived dissolved organic matter is due to the physicochemical properties (K_{OC}) possible (Zubrod et al. 2019). Those interactions are, however, beyond the scope of this manuscript but open an interesting avenue to deepen the scientific understanding on fungicide toxicity in complex (eco)systems (Eriksson et al. 2004; Hassett 2006; Arias-Estévez et al. 2008).

Nevertheless, since the hyporheic zone is a mixing and transition zone of organic matter, nutrients, and pollutants (reviewed by Krause et al. 2011), effects of fungicides on microbial leaf decomposition could even propagate to groundwater or surface water habitats. For example, the hyporheic zone provides shelter from predation and environmental conditions (Schwoerbel 1967; Williams and Hynes 1974; Stubbington 2012) many species of higher trophic level (e.g., macroinvertebrates) spend at least part of (i.e., stygophiles) or their lifecycle (i.e., stygobites) below the sediment–water interface (Gibert 1994). Likewise, indirect effects on life-history traits of detritus-based stygophiles could propagate to the benthic zone via migration and subsequent predation. Such trophic cascades (cf. Cedergreen and Rasmussen 2017) have been observed in a benthic setting, since fungi influence life-history traits of detrivores (Chung and

Suberkropp 2009) and thus the energy and nutrient transfer to higher trophic levels (Likens et al. 2009).

Conclusion

As hypothesized, our study shows a considerable decrease in microbial functioning in the hyporheic zone compared to the benthic zone. Although there are gaps in the mechanistic understanding, there was a twofold higher fungicide effect in the fine sediment on microbial functioning compared to the benthic zone.

Further research on this topic seems all the more important given the projected increases in pesticide use (including fungicides) due to climate change and agricultural expansion (Tilman 2001; Stocker et al. 2013). In this context, our study underlines the need to consider the hyporheic zone, and thus the major storage site of organic matter in streams (Metzler and Smock 1990) to gain a comprehensive understanding of chemical stress effects on carbon and energy cycling in detritus-based freshwater ecosystems.

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Conflict of Interest

None declared.

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