Impact of fungicides on an aquatic decomposer-detritivore system

Auswirkungen von Fungiziden auf ein aquatisches Zersetzer-Detritivoren-System

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DECLARATION

I hereby declare that I independently conducted the work presented in this thesis entitled “Impact of fungicides on an aquatic decomposer-detritivore system”. All used assistances are mentioned and involved contributors are either co-authors of or are acknowledged in the respective publication. In all cases, I designed and planed the studies, conducted the experiments, performed the associated analyses, evaluated the data, and wrote the respective publication – with support of the named persons.

This thesis has never been submitted elsewhere for an examination, as a thesis or for evaluation in a similar context to any department of this university or any scientific institution. I am aware that a violation of the aforementioned conditions can have legal consequences.

Landau, December 23rd, 2015

Place, Date Jochen P. Zubrod

The following parts of this thesis are published in peer-reviewed international journals:

- **APPENDIX A.1:** Zubrod, J. P., Baudy, P., Schulz, R., Bundschuh, M., 2014. Effects of current-use fungicides and their mixtures on the feeding and survival of the key shredder *Gammarus fossarum*. Aquatic Toxicology 150, 133-143.
- **APPENDIX A.2:** Zubrod, J. P., Feckler, A., Englert, D., Koksharova, N., Rosenfeldt, R. R., Seitz, F., Schulz, R., Bundschuh, M., 2015. Inorganic fungicides can increase microorganism-mediated palatability of leaves for a shredder but reduce microbial leaf decomposition. Journal of Applied Ecology 52, 310-322.
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1. ABSTRACT

Leaf litter breakdown is a fundamental process in aquatic ecosystems, being mainly mediated by decomposer-detritivore systems that are composed of microbial decomposers and leaf-shredding, detritivorous invertebrates. The ecological integrity of these systems can, however, be disturbed, amongst others, by chemical stressors. Fungicides might pose a particular risk as they can have negative effects on the involved microbial decomposers but may also affect shredders via both waterborne toxicity and their diet; the latter by toxic effects due to dietary exposure as a result of fungicides' accumulation on leaf material and by negatively affecting fungal leaf decomposers, on which shredders' nutrition heavily relies. The primary aim of this thesis was therefore to provide an in-depth assessment of the ecotoxicological implications of fungicides in a model decomposer-detritivore system using a tiered experimental approach to investigate (1) waterborne toxicity in a model shredder, i.e., *Gammarus fossarum*, (2) structural and functional implications in leaf-associated microbial communities, and (3) the relative importance of waterborne and diet-related effects for the model shredder. Additionally, knowledge gaps were tackled that were related to potential differences in the ecotoxicological impact of inorganic (also authorized for organic farming in large parts of the world) and organic fungicides, the mixture toxicity of these substances, the field-relevance of their effects, and the appropriateness of current environmental risk assessment (ERA).

In the course of this thesis, major differences in the effects of inorganic and organic fungicides on the model decomposer-detritivore system were uncovered; e.g., the palatability of leaves for *G. fossarum* was increased by inorganic fungicides but deteriorated by organic substances. Furthermore, non-additive action of fungicides was observed, rendering mixture effects of these substances hardly predictable. While the relative importance of the waterborne and diet-related effect pathway for the model shredder seems to depend on the fungicide group and the exposure concentration, it was demonstrated that neither path must be ignored due to additive action. Finally, it was shown that effects can be expected at field-relevant fungicide levels and that current ERA may provide insufficient protection for decomposer-detritivore systems. To safeguard aquatic ecosystem functioning, this thesis thus recommends including leaf-associated microbial communities and long-term feeding studies using detritus feeders in ERA testing schemes, and identifies several knowledge gaps whose filling seems mandatory to develop further reasonable refinements for fungicide ERA.

2. ZUSAMMENFASSUNG

Der Abbau von Laubstreu stellt in aquatischen Ökosystemen einen fundamentalen Prozess dar, der hauptsächlich durch Zersetzer-Detritivoren-Systeme bewirkt wird, welche sich aus mikrobiellen Zersetzern und laubzerkleinernden, detritivoren Invertebraten (=Shredder) zusammensetzen. Die ökologische Integrität dieser Systeme kann aber unter anderem durch Umweltchemikalien gestört werden. Fungizide könnten in diesem Zusammenhang ein besonderes Risiko darstellen, da sie negative Effekte auf die beteiligten mikrobiellen Zersetzer haben, aber auch Shredder durch Exposition über die Wasserphase und nahrungsbedingte Effekte beeinflussen können; letzteres bedingt zum einen durch die Akkumulation von Fungiziden auf Laubmaterial und der damit potentiell einhergehenden Exposition über die Nahrung und zum anderen durch die negative Beeinträchtigung laubzersetzender Pilze, die in der Ernährung von Shreddern eine wichtige Rolle spielen. Das primäre Ziel dieser Arbeit war daher eine umfassende Untersuchung der ökotoxikologischen Implikationen von Fungiziden in einem Modell-Zersetzer-Detritivoren-System. Dabei kam ein gestuftes Testverfahren zur Untersuchung (1) der Effekte auf einen Modellshredder (*Gammarus fossarum*) aufgrund von Exposition über die Wasserphase, (2) der strukturellen wie funktionellen Implikationen in laubassoziierten mikrobiellen Gemeinschaften und (3) der relativen Bedeutung von wasserexpositions- und nahrungsbedingten Effekten auf den Modellshredder zum Einsatz. Zusätzlich sollten Wissenslücken hinsichtlich potentieller Unterschiede des ökotoxikologischen Einflusses anorganischer (in weiten Teilen der Welt zur Nutzung im ökologischen Landbau zugelassen) und organischer Fungizide, der Mischungstoxizität dieser Substanzen, der Umweltrelevanz ihrer Effekte sowie der Zweckmäßigkeit der aktuellen Umweltrisikobewertung geschlossen werden.

Im Rahmen dieser Arbeit wurden bedeutende Unterschiede bezüglich der Effekte anorganischer und organischer Fungizide auf das Modell-Zersetzer-Detritivoren-System aufgedeckt; so wurde beispielsweise die Schmackhaftigkeit von Laub für *G. fossarum* durch anorganische Fungizide positiv, durch synthetische aber negativ beeinflusst. Zudem konnte gezeigt werden, dass Fungizide nicht-additiv wirken können, was die Vorhersage von Mischungseffekten erschwert. Des Weiteren scheint die relative Bedeutung von wasserexpositions- und nahrungsbedingten Effekten für den Modell-Shredder zum einen von der Fungizidgruppe und zum anderen von der Expositionskonzentration abzuhängen. Da die genannten Effektpfade aber additiv wirken, müssen zur Bewertung des Gesamteffekts beide

Beachtung finden. Weiterhin zeigte sich, dass bereits bei umweltrelevanten Fungizidkonzentrationen Effekte auf Zersetzer-Detritivoren-Systeme zu erwarten sind und die derzeitige Umweltrisikobewertung nur ein unzureichendes Maß an Schutz für diese Organismenkomplexe gewährleistet. Um die Funktionsfähigkeit aquatischer Systeme sicherzustellen, geht aus dieser Arbeit daher die Empfehlung hervor, die Testung von laubassoziierten mikrobiellen Gemeinschaften und Langzeit-Fütterungsversuche mit Detritus-Konsumenten standardmäßig in die Umweltrisikobewertung von Fungiziden zu integrieren. Zudem wird auf mehrere Wissenslücken hinsichtlich Fungizideffekten in Zersetzer-Detritivoren-Systemen verwiesen, deren Schließung die Grundlage für weitere Anpassungen der Fungizid-Umweltrisikobewertung schaffen würde.

3. INTRODUCTION

3.1 BACKGROUND

The application of fungicides is considered essential to secure global food supply (Strange and Scott, 2005) since despite current management practices, the portion of crop yield lost annually to fungal pathogens is estimated to be sufficient to feed more than 8% of the world's human population (Fisher et al., 2012). This also holds true for organic farming (Wilbois et al., 2009), for which – in contrast to conventional farming – organic fungicides (such as triazoles and strobilurines that inhibit fungal sterol production and mitochondrial respiration, respectively; Bartlett et al., 2002; Bodey, 1992) are not available. But inorganic substances (particularly copper (Cu) and elemental sulfur (S) that interact with, amongst others, proteins and affect mitochondrial respiration, respectively; Flemming and Trevors, 1989; Williams and Cooper, 2004) are also authorized for this form of agriculture in large parts of the world including the U.S. (USDA, 2015) and many European countries (e.g., BVL, 2015a). Through point (e.g., wastewater treatment plant outlets; Kahle et al., 2008) and diffuse (e.g., surface run-off; Bereswill et al., 2012) sources, fungicides can enter surface water bodies following their application. There, a large range of these substances is frequently detected in mixtures (e.g., Battaglin et al., 2010; Bereswill et al., 2012; Fernández et al., 2015; Süss et al., 2006). This is a consequence of their prophylactic application (with up to ten applications per season; Reilly et al., 2012) and the multitude of used active ingredients (e.g., more than 120 approved for use in the European Union as of September 2015; EC, 2015), which is partly owing to resistance management (Staub and Sozzi, 1984). As a result, fungicide exposure may contribute to ecologically meaningful changes in aquatic communities and ecosystem functions such as leaf litter breakdown in the field (cf. Fernández et al., 2015; Schäfer et al., 2012).

In small, low-order streams, allochthonous organic material such as leaf litter may contribute up to 99% of the total annual energy input (Fisher and Likens, 1973). This terrestrial subsidy can therefore, despite its low quality, drive in-stream microbial respiration and is of vital importance for invertebrate secondary production (Marcarelli et al., 2011). Accordingly, its breakdown is mainly mediated by decomposer-detritivore systems (e.g., Hieber and Gessner, 2002) that are composed of microbial decomposers and leaf-shredding, detritivorous macroinvertebrates (i.e., shredders; Cummins and Klug, 1979). The involved microorganisms, particularly the polyphyletic group of fungi subsumed as aquatic hyphomycetes (Suberkropp and

Klug, 1976), accomplish two fundamental functions: first, they contribute considerably to the processing of leaves by direct decomposition (e.g., Hieber and Gessner, 2002; Taylor and Chauvet, 2014). Second, they condition leaf material by increasing the leaves' nutrient content (e.g., proteins and lipids) and degrading recalcitrant structural leaf components – transformations that convert leaves into a more palatable and nutritious food source for shredders (Bärlocher, 1985). These macroinvertebrates in turn act as a key link in detrital food webs since they produce fine particulate organic matter (e.g., fecal pellets), the predominant food resource for collecting species (Bundschuh and McKie, in press; Cummins and Klug, 1979), and represent an important prey for aquatic and terrestrial (in)vertebrate predators (e.g., MacNeil et al., 1999). The pivotal role of decomposer-detritivore systems for local communities, but also for those situated up to tens of kilometers downstream of the headwaters (Webster, 2007), is emphasized by the strong bottom-up regulation of the food webs inhabiting detritus-based stream ecosystems (Wallace and Eggert, 1997).

The ecological integrity of decomposer-detritivore systems may, however, be affected by fungicides since these substances are designed to act on fungal organisms. Thus, these pesticides may also have the potential to affect non-target fungi such as aquatic hyphomycetes. While a few older studies investigating the sensitivity of single species of these fungi towards fungicides (Chandrashekar and Kaveriappa, 1989; 1994; Miersch et al., 1997) only detected negative implications at relatively high concentrations (in the mg/L range or higher), more recent investigations on the community level using single demethylation-inhibiting fungicides (Artigas et al., 2012; Bundschuh et al., 2011; Dimitrov et al., 2014; Flores et al., 2014; Zubrod et al., 2011) or field exposures (Fernández et al., 2015) suggest much lower effect threshold concentrations (i.e., in the µg/L range).

On the other hand, shredders may also be affected by these pesticides via two pathways, whose combination can be assumed to be the realistic worst case for these macroinvertebrates (cf. Zubrod et al., 2011): first, many antifungal substances act on biological processes that are highly conserved (such as energy production and cell division) and thus not specific to fungi (Stenersen, 2004). Thus, fungicides can impact a range of different taxonomic groups of aquatic organisms (Maltby et al., 2009), resulting in the potential for toxic effects in shredders when subjected to waterborne exposure towards these substances. In this context, implications in leaf breakdown via feeding of the amphipod shredder genus *Gammarus* were already demonstrated for Cu (e.g., Tattersfield, 1993) and the azole fungicide tebuconazole

(Zubrod et al., 2010). Second, fungicides have the potential to affect shredders via their diet; on the one hand, via accumulation on leaf material (Dimitrov et al., 2014), which may also cause toxic effects when fungicides are co-ingested with the leaf substrate as already reported for other pesticides (e.g., Bundschuh et al., 2013). On the other hand and as stated above, fungicides can negatively affect fungal leaf decomposers, which in turn indicates detrimental effects on microbial conditioning and thus food quality of leaf material for shredders (Bundschuh et al., 2011).

However, information about the relative importance of both the waterborne and diet-related pathways for shredders is scarce: *Echinogammarus berilloni* CATTA (Crustacea: Amphipoda) had a lower leaf consumption and fitness induced by the fungicide imazalil only if waterborne and diet-related pathways acted in combination (Flores et al., 2014). Yet, as the latter observations were reported at a fungicide concentration causing substantial mortality in the shredder, the observed sublethal effects likely induced by the diet-related pathway seem comparably unimportant. In contrast, growth and lipid content of *G. fossarum* KOCH (Crustacea: Amphipoda) were reduced when fed leaves microbially conditioned in the presence of tebuconazole and being exposed to the same fungicide via the water phase (Zubrod et al., 2011). While the applied fungicide concentration resulted in a deteriorated leaf-associated fungal community, it was unlikely to trigger any waterborne toxicity (Zubrod et al., 2010). Therefore, this study provided indirect evidence for the effects being mediated by the diet-related effect pathway.

Despite these inconsistences, the few studies available indeed indicate fungicide implications in decomposer-detritivore systems (e.g., Fernández et al., 2015). This highlights the paramount importance of a more in-depth understanding of fungicide effect paths within these organism complexes and a general thorough assessment of the ecological risks associated with the introduction of these substances into aquatic systems (cf. EFSA, 2013a; Maltby et al., 2009), which seem mandatory to safeguard the integrity of the key ecosystem function of leaf litter breakdown.

3.2 MOTIVATION AND OBJECTIVES

Despite the indicated risk for decomposer-detritivore systems posed by fungicides, effects of these substances on organisms and processes involved in leaf litter breakdown remain poorly studied and understood. Generally, there is a lack of data on fungicide effects (particularly at field-relevant levels) on leaf-associated microorganisms and shredders resulting in the impossibility of judging the level of protection granted by current European environmental risk assessment (ERA) practices for these organisms and the functions they provide (cf. Maltby et al., 2009). Furthermore, several more specific knowledge gaps await scientific assessment: first of all, a separate ecotoxicological analysis of inorganic as well as organic fungicides, and thus an estimation of the ecological implications associated with the fungicide use in organic (only inorganic fungicides authorized) and conventional (both groups authorized) farming, is lacking so far. Moreover, the joint toxicity of fungicides has hardly been studied although these substances are commonly detected as mixtures (e.g., Battaglin et al., 2010; Bereswill et al., 2012; Reilly et al., 2012), and a better understanding would for instance aid in the interpretation of field studies (Fernández et al., 2015). Finally, the state of knowledge regarding fungicide-induced effects that propagate to other trophic levels is insufficient, particularly regarding physiological and functional effects in shredders due to consumption of fungicide-affected leaf material (i.e., diet-related path) and the importance of this pathway compared to waterborne toxicity.

The primary aim of this thesis is thus to provide an in-depth assessment of the ecotoxicological implications of inorganic and organic fungicides in a model decomposer-detritivore system (see chapter 3.3) using a tiered experimental approach (see chapter 3.4) to answer four main research questions that are supposed to tackle the knowledge gaps identified above:

1. Do inorganic and organic fungicides generally differ regarding their ecotoxicological effects?
2. Do fungicides interact and their mixtures thus result in effects that are not predictable from single-substance experiments?
3. What is the relative importance of waterborne toxicity and diet-related effects for shredders?
4. Do environmentally relevant fungicide concentrations trigger ecotoxicological effects in aquatic decomposer-detritivore systems and do current European ERA practices provide a sufficient level of protection for these systems?

3.3 MODEL SYSTEM

To answer these main research questions, this thesis made use of a selection of current-use fungicides. As inorganic fungicides, three Cu-based fungicides (to assess for comparable effect patterns due to their common major toxic cations; Flemming and Trevors, 1989) and S were assessed. To represent the large group of organic fungicides, five substances covering a cross-section of modes of toxic action, applied in the field, were chosen (Table 3.1).

Table 3.1. Classification and mode of toxic action of the assessed fungicides.

Fungicide	Chemical group	Chemical family	Mode of action[a]
Cu-hydroxide	Inorganic	Inorganica	Multi-site
Cu-octanoate		Inorganica	Multi-site
Cu-sulfate		Inorganica	Multi-site
S		Inorganica	Multi-site
Azoxystrobin	Organic	Strobilurins	Inhibition of mitochondrial respiration
Carbendazim		Benzimidazoles	Inhibition of mitosis and cell division
Cyprodinil		Anilino-pyrimidines	Inhibition of amino acid and protein synthesis
Quinoxyfen		Quinolines	Perturbation of signal transduction
Tebuconazole		Triazoles	Inhibition of sterol biosynthesis

[a] FRAC (2015)

Moreover, during this thesis, a model decomposer-detritivore system (e.g., Bundschuh et al., 2009) was assessed that included a natural leaf-associated microbial community, which was sampled at the Rodenbach near Grünstadt, Germany (49°33'N; 8°02'E), whenever necessary. This approach was favored over using a defined community of a few laboratory-reared species of aquatic hyphomycetes (e.g., Pascoal et al., 2010) to mirror the complexity of natural leaf-associated microbial communities and thus to cover all species interactions that may modulate fungicide effects in these communities (cf. Brandt et al., 2015; Bundschuh et al., 2011). As a model shredder, *G. fossarum* (cryptic lineage B; Feckler et al., 2014), sampled at the Hainbach, Germany (49°14'N; 8°03'E), was used. *Gammarus* spp. generally appear as sensible test species for fungicide-induced waterborne toxicity and diet-related effects based on their ecological significance (Dangles et al., 2004; Englert et al., 2013), their well-documented sensitivity against (chemical) stressors (e.g., Maltby et al., 2002), and their highly selective food choice (Arsuffi and Suberkropp, 1989; Bundschuh et al., 2009).

3.4 THESIS LAYOUT

This thesis was subdivided in three experimental phases (Fig. 3.1). To verify adequate dosing of the model stressors, each phase was accompanied by a thorough assessment of waterborne fungicide exposure using an inductively coupled plasma quadrupole mass spectrometry (ICP-MS; for Cu-based fungicides; Rosenfeldt et al., 2014) and an ultra-high performance liquid chromatography mass spectrometry (for organic fungicides; Zubrod et al., 2011) system.

The first phase aimed at assessing waterborne toxicity of the model fungicides towards *G. fossarum*. The animals were exposed via the water phase towards single inorganic and organic fungicides using a 6-day long, semi-static and a 7-day long, static test design (Zubrod et al., 2010), respectively. During the experiments, mortality as well as the test organisms' feeding activity – a sensitive, robust and ecologically relevant ecotoxicological endpoint (Maltby et al., 2002) – were recorded. Based on the results of these single-substance trials, two mixture experiments, using an "A in the presence of B" and a "fixed ratio" design (Jonker et al., 2011), were conducted. These were used to assess the compliance of the effects of a binary mixture of Cu and S as well as one mixture comprising all five organic model fungicides with the predictions of the reference model "independent action" (IA). This reference model assumes that mixture components have dissimilar modes of toxic action (Bliss, 1939) as can be assumed for the assessed mixtures (Table 3.1).

During the second phase, leaf material that was microbially colonized for 12 days under control conditions or in the presence of inorganic or organic fungicides was used to assess the two important functions provided by leaf-associated microorganisms: i.e., microbial leaf decomposition as well as leaf palatability (=diet-related effect pathway), the latter being studied using the food choice of *Gammarus* (Bundschuh et al., 2009; 2011). Additionally, to facilitate a mechanistic understanding of functional effects, leaf-associated microbial communities were characterized: aquatic hyphomycete community composition was determined by spore morphology (Bärlocher, 1982; Pascoal and Cássio, 2004), leaves' ergosterol content was quantified as a proxy for leaf-associated fungal biomass (Gessner and Schmitt, 1996), and bacterial densities on the leaves were assayed via epifluorescence microscopy (Buesing, 2005). To assess the potential impact of a repellent effect caused by adsorbed fungicides on the food choice of *Gammarus* (cf. Hahn and Schulz, 2007; Rasmussen et al., 2012), further experiments were performed that excluded any fungicide-induced changes in the leaf-associated microbial

communities. Therefore, fungicide adsorption to the leaf material was simulated for the lowest concentration triggering a significant food-choice response in the main experiments (or the highest concentration if no significant effect was found) using pre-conditioned, autoclaved leaf discs. These experiments were, however, only conducted for organic fungicides since an adsorption-related explanation for gammarids' feeding patterns in response to Cu is unlikely (Tattersfield, 1993). Again, after assessing the model fungicides singly, the same mixtures as in the first phase were investigated using an "A in the presence of B" and a "fixed-concentration ratio" design (Jonker et al., 2011) for inorganic and organic fungicides, respectively.

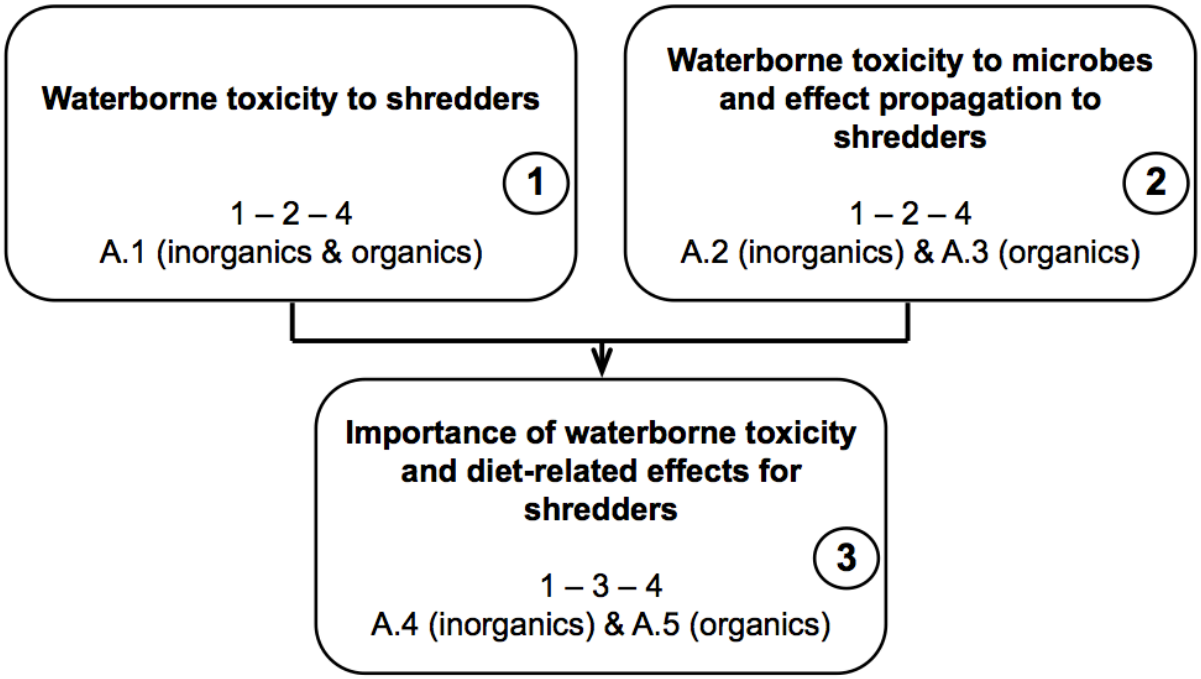

Fig. 3.1. Scheme displaying the three phases of this thesis including their main objective (box title), the research questions that were tackled (numbers separated by dashes; see chapter 3.2), and by which publication **[Appendix A.1-A.5]** the respective fungicide group is covered.

Finally and based on the effect thresholds regarding the two effect pathways determined during the preceding two phases, two experiments were designed for the third stage of this thesis. These made use of a full 2x2-factorial design to rigorously test the relative importance of waterborne toxicity and diet-related effects of Cu and the organic model fungicide mixture. The first factor assessed was the absence or presence of the respective fungicide (mixture) during a 12-day long microbial conditioning of leaf material (used as food for *Gammarus*), while the second factor was the absence or presence of the fungicides in the medium used for culturing the shredders during a 24-day long bioassay (Zubrod et al., 2011). To understand potential diet-related effects in gammarids, both leaf-associated microbial

communities (see above) as well as fungicide adsorption on leaf material (only for Cu by means of an aqua regia digestion followed by quantification using ICP-MS) were characterized. Furthermore, the consequences of waterborne toxicity and diet-related effects of the fungicides in the test animals were assessed by quantifying their energy processing (leaf consumption and feces production) as well as their physiological fitness, which was evaluated using growth and lipid content (Van Handel, 1985). The results gained during the three experimental phases, which were part of this cumulative thesis, were published in five peer-reviewed journal articles (Fig. 3.1) **[Appendix A.1-A.5]**.

4. RESULTS

4.1 WATERBORNE TOXICITY TOWARDS SHREDDERS

During the single-substance experiments, S (up to almost 5 mg/L) was the only fungicide tested for which neither lethal nor sublethal effects on *G. fossarum* could be detected (Table 4.1) **[Appendix A.1]**. The Cu-compounds, in contrast, were of high lethal toxicity and featured comparable threshold concentrations for effects on gammarids' survival (Table 4.1). But they differed markedly with regards to their sublethal toxicity (i.e., feeding inhibition) with only Cu-sulfate showing sublethal effects at concentrations triggering no substantial mortality (i.e., $EC_{20}<LC_{20}$; effective and lethal concentrations resulting in 20% inhibition of gammarids' feeding rate and mortality, respectively; Table 4.1). On the contrary, EC_{50}-values of organic fungicides for gammarids' feeding activity were generally observed at concentrations resulting in less than 20% mortality, with the exception of carbendazim showing high lethal toxicity ($LC_{50}<EC_{50}$; Table 4.1) **[Appendix A.1]**.

Table 4.1. EC- and LC-values derived from bioassays with single fungicides or mixtures. Concentrations are given in µg/L or µg Cu/L and are supplemented by 95% confidence intervals (CIs) **[Appendix A.1]**.

Fungicide	EC_{20}	EC_{50}	LC_{20}	LC_{50}
Cu-hydroxide	65.6 (0.0-426.7)	-	58.7 (48.1-69.4)	89.1 (81.3-96.9)
Cu-octanoate	85.2 (58.5-111.8)	123.2 (100.7-145.7)	60.0 (49.0-71.0)	91.1 (82.0-100.2)
Cu-sulfate	36.4 (21.7-50.0)	67.9 (46.5-89.4)	66.2 (62.0-70.4)	77.8 (73.6-81.9)
S	-	-	-	-
Azoxystrobin	79.0 (30.2-127.7)	90.8 (79.1-102.5)	98.0 (70.4-125.6)	148.4 (127.8-169.1)
Carbendazim	32.5 (14.3-50.7)	75.0 (47.5-102.6)	35.3 (13.5-57.1)	51.0 (43.6-58.5)
Cyprodinil	15.7 (1.3-30.2)	50.5 (0.0-103.3)	73.9 (13.2-134.6)	-
Quinoxyfen	6.0 (0.1-12.1)	23.8 (11.5-36.1)	-	-
Tebuconazole	91.7 (38.6-144.8)	197.8 (144.1-251.6)	-	-
Inorganic mixture	48.8 (30.0-67.6)	-	72.2 (61.7-82.7)	96.7 (90.6-102.8)
Organic mixture	60.6 (32.3-89.0)	119.5 (97.3-141.6)	-	-

Despite the absence of effects when applied singly, S substantially reduced Cu-sulfate's (tested because of its high sublethal toxicity; Table 4.1) effects on survival (Fig. 4.1a) and feeding (Fig. 4.1b) of *G. fossarum* when applied in a binary mixture using an "A in the presence of B" design **[Appendix A.1]**. The observed antagonism (statistically significant non-compliance with IA is indicated when predictions are not included in 95% CIs of observations; Fig. 4) increased Cu-sulfate's LC_{50} by more

than 20% compared to the single-substance experiment, while feeding inhibition did not exceed 38% effect even at the highest Cu-concentration (Table 4.1; Fig. 4.1b).

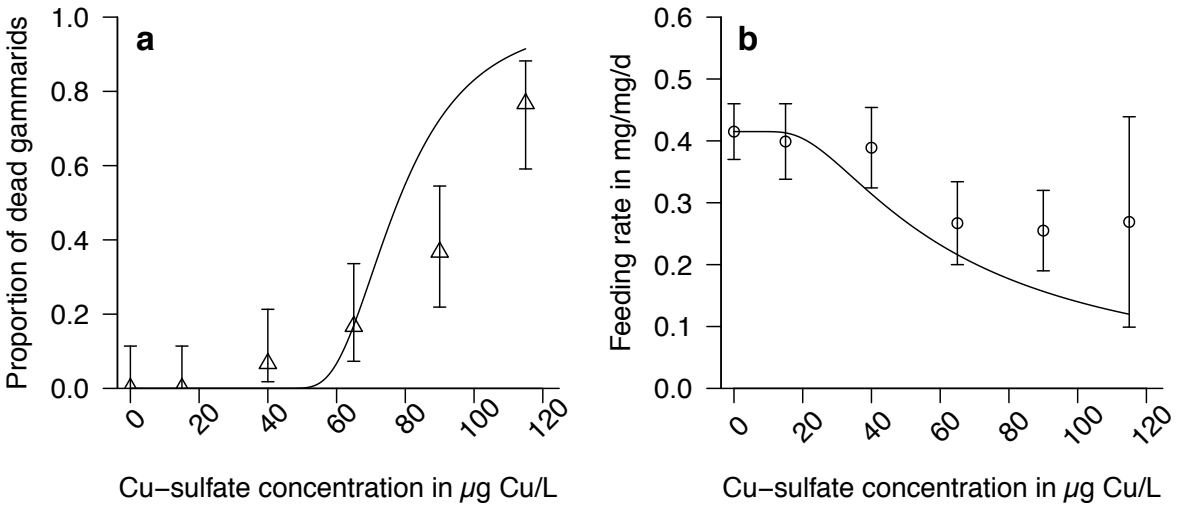

Fig. 4.1. Proportion of dead animals (triangles; a) and mean feeding rates (circles; b) of *G. fossarum* (with 95% CIs) when exposed to different concentrations of Cu-sulfate with a fixed concentration of S (165 µg/L). The lines indicate predictions by IA **[Appendix A.1]**.

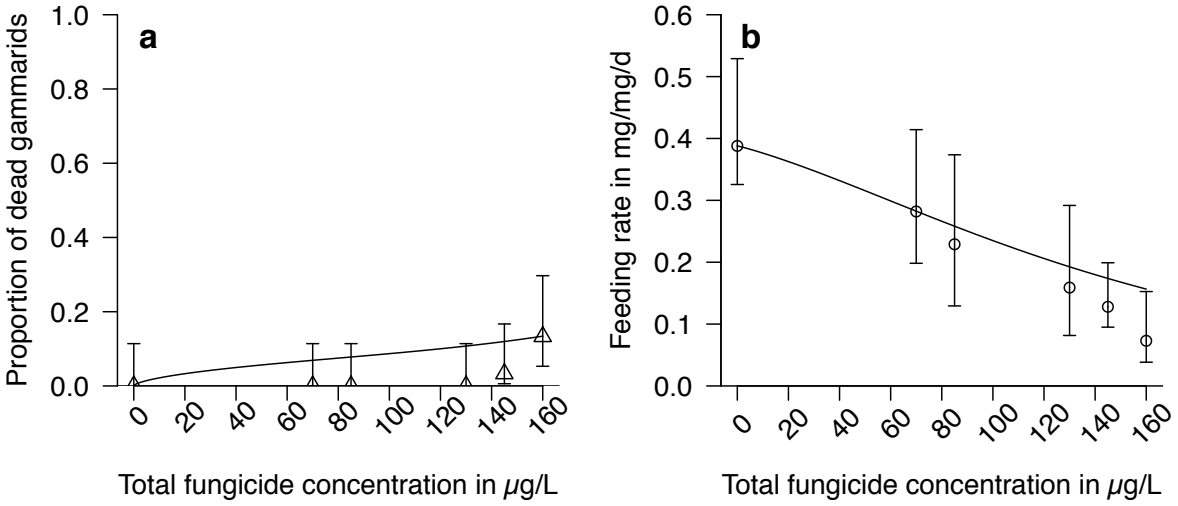

Fig. 4.2. Proportion of dead animals (triangles; a) and median feeding rates (circles; b) of *G. fossarum* (with 95% CIs) when exposed to different concentrations of the organic mixture. The lines indicate predictions by IA **[Appendix A.1]**.

The organic fungicide mixture caused no statistically significant deviation from IA with regard to mortality (Fig. 4.2a) **[Appendix A.1]**. Moreover, the observed feeding rate of gammarids exposed to this mixture agreed well with the prediction by IA for the lowest total fungicide concentration (70 µg/L; Fig. 4.2b). At higher concentrations, however, observed feeding rates were lower than predicted by IA. The deviation was statistically significant only at the highest tested mixture concentration (160 µg/L) with the observed feeding rate inhibition compared to the control deviating by approximately 35% from that predicted by the reference model.

4.2 WATERBORNE TOXICITY TOWARDS MICROBES AND EFFECT PROPAGATION TO SHREDDERS

All tested inorganic fungicides reduced microbial leaf decomposition by up to 30%, with significant effects observed at concentrations ≥10 µg Cu/L and ≥800 µg/L for Cu and S, respectively (statistical tests used for and *P*-values resulting from null-hypothesis significance testing are reported in the respective appendices) **[Appendix**

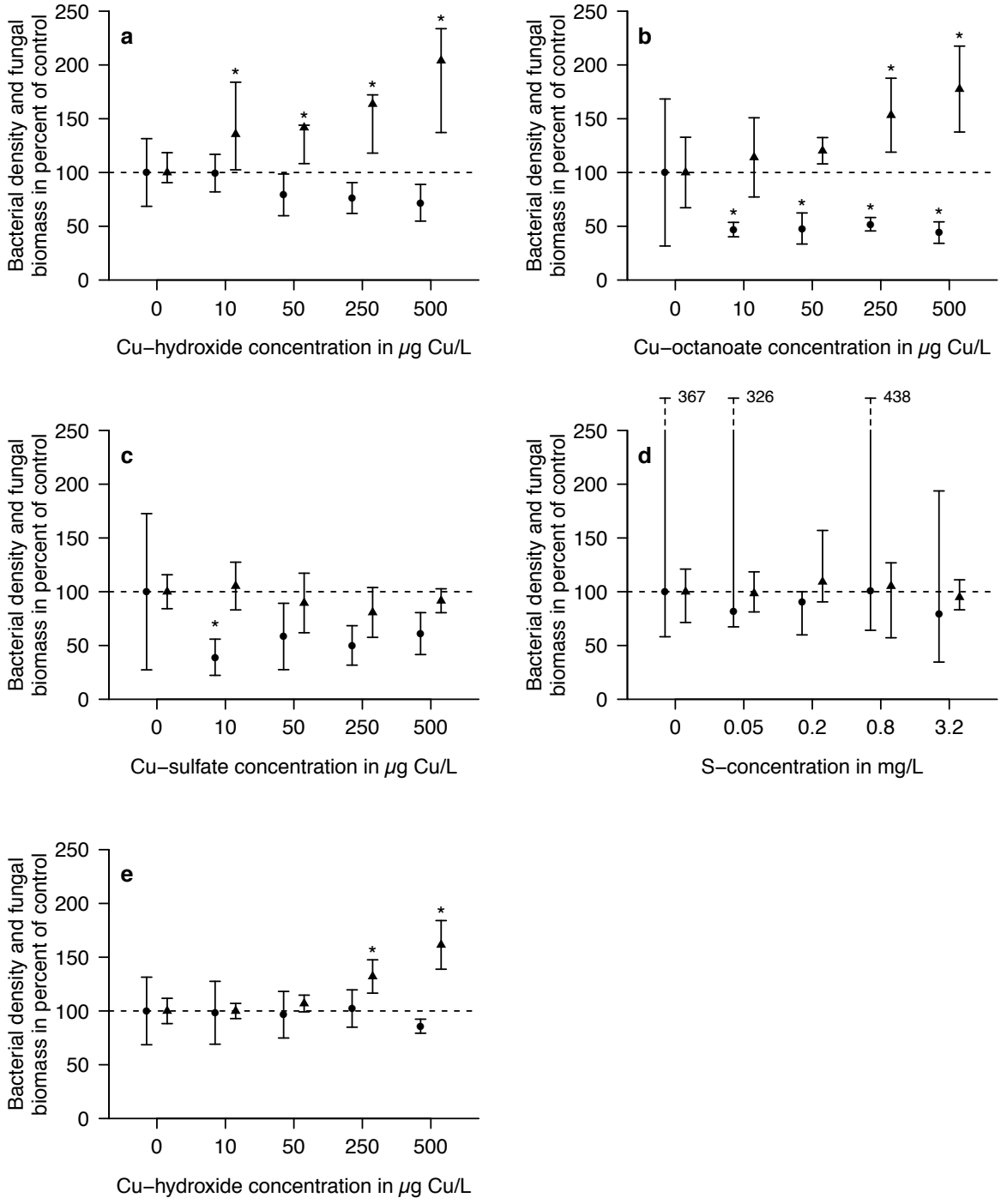

Fig. 4.3. Mean or median (with 95% CI) bacterial density (circles) and fungal biomass (triangles) relative to the respective control associated with leaf material conditioned in the presence of different concentrations of a) Cu-hydroxide, b) Cu-octanoate, c) Cu-sulfate, d) S, and e) Cu-hydroxide amended by 0.2 mg S/L. Asterisks denote statistically significant differences to the respective control **[Appendix A.2]**.

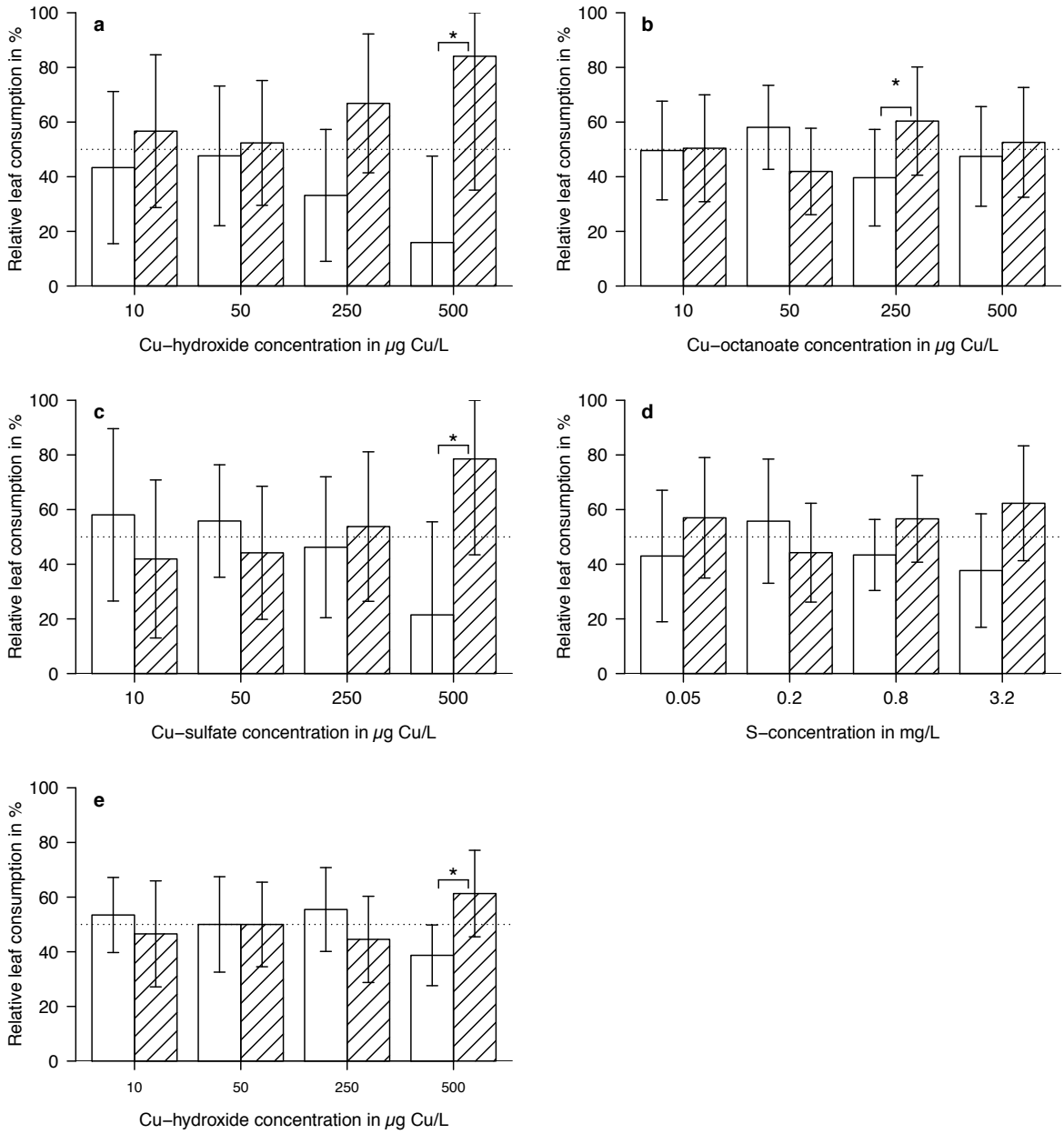

Fig. 4.4. Mean relative consumption (with 95% CI) of *G. fossarum* on leaf material conditioned either under control conditions (white bars) or in the presence of different concentrations of a) Cu-hydroxide, b) Cu-octanoate, c) Cu-sulfate, d) S, and e) Cu-hydroxide amended by 0.2 mg S/L (hatched bars). Asterisks denote statistically significant differences. While 100% is the total leaf consumption in a food-choice experiment, the horizontal dotted line indicates the no-effect level (50% consumption of both leaf types) **[Appendix A.2]**.

A.2]. The Cu-induced functional effects went along with significant reductions in bacterial densities by up to 60%, significant increases in fungal biomass by up to 100% (both at ≥10 µg Cu/L; Fig. 4.3a-c) as well as a significantly altered fungal community composition (at 50 µg Cu/L for Cu-octanoate and 500 µg Cu/L for Cu-hydroxide and Cu-sulfate) **[Appendix A.2]**. In contrast, S did not result in any significant structural changes in leaf-associated microbial communities (Fig. 4.3d) **[Appendix A.2]**. Consistent with these findings, S did not significantly alter the leaf

preference of *G. fossarum* over all concentrations tested (Fig. 4.4d), while leaf material exposed to Cu-concentrations of 250 µg Cu/L (for Cu-octanoate) and 500 µg Cu/L (for Cu-hydroxide and Cu-sulfate) was significantly preferred over unexposed leaves (Fig. 4.4a-c). In line with the observations during the first phase, a binary mixture of Cu-hydroxide (applied as the Cu-compound with the strongest effect on fungal biomass; Fig. 4.3a-c) and S resulted in significantly lower effect sizes for nearly all assessed endpoints or even caused reversed effect directions compared to the single-substance experiment with Cu-hydroxide (Figs. 4.3e and 4.4e) **[Appendix A.2]**.

As observed for Cu and S, microbial leaf decomposition was significantly negatively affected by the organic fungicides azoxystrobin (at 100 and 500 µg/L), carbendazim (at ≥245 µg/L), and cyprodinil (at ≥200 µg/L), while quinoxfen did not significantly affect this endpoint and tebuconazole even siginificantly increased decomposition at 1 µg/L by ~10% **[Appendix A.3]**. Similarly, observations for bacterial densities were inconsistent, being only significantly negatively affected by tebuconazole **[Appendix A.3]**. Likewise, fungal biomass was either not significantly affected (carbendazim), significantly reduced (azoxystrobin, cyprodinil, and at high tebuconazole concentrations), or significantly increased (at low to intermediate concentrations of quinoxyfen and tebuconazole) as a result of fungicide exposure **[Appendix A.3]**. The only structural changes consistently observed for all organic fungicides except quinoxyfen were significant or near-significant (cyprodinil) changes in fungal community composition (Fig. 4.5) and significant reductions in fungal species richness **[Appendix A.3]**. Also, all tested organic fungicides affected leaf palatability negatively, which was displayed by a statistically significant preference of *Gammarus* for control over fungicide-exposed leaves at the highest concentrations of carbendazim, cyprodinil, quinoxyfen, and tebuconazole (Fig. 4.6b-e). Moreover, similar trends were observed at lower concentrations of these substances and at the highest concentration of azoxystrobin (Fig. 4.6a). The add-on experiments that excluded any fungicide-induced changes in the leaf-associated microbial communities but simulated adsorption of the fungicides revealed effect sizes for gammarids' food choice very similar to those observed in the main experiments (Fig. 4.6) **[Appendix A.3]**. Moreover, during the mixture experiment, despite observing significant reductions in the number of leaf-associated fungal species and bacterial density at the lowest applied sum concentration (i.e., 6 µg/L), a large fraction of the joint effects of the organic mixture was lower than predicted by IA **[Appendix A.3]**.

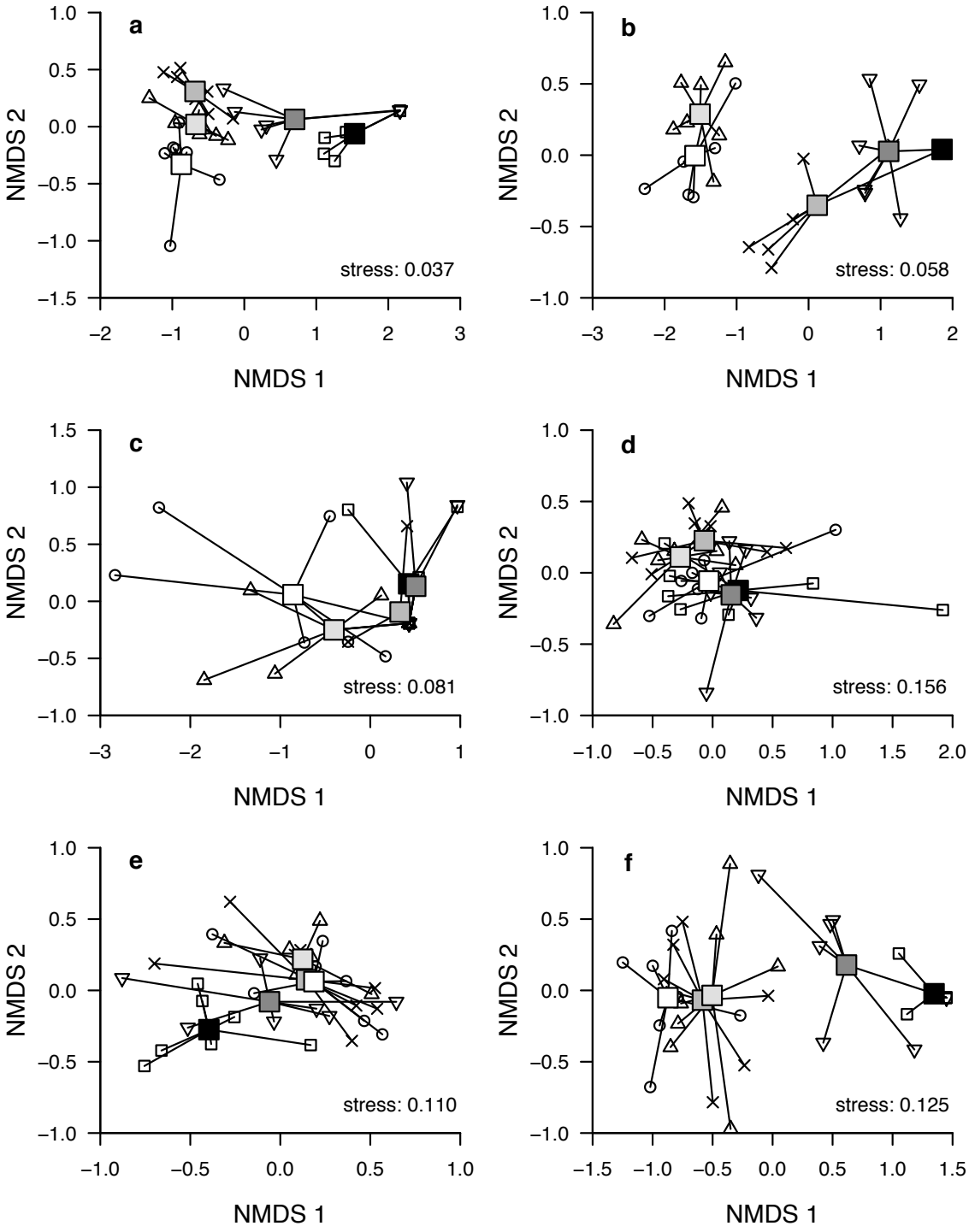

Fig. 4.5. Non-metric multidimensional scaling (NMDS; an ordination technique to display the dissimilarities of samples; Clarke, 1993) plots of aquatic hyphomycete communities associated with leaf material conditioned in the presence of different concentrations of a) azoxystrobin, b) carbendazim, c) cyprodinil, d) quinoxyfen, e) tebuconazole, and f) the fungicide mixture. Symbols are (from lowest to highest concentration): circles (i.e., control), triangles, crosses, inverted triangles, and squares. Additionally, concentrations (see Table 1 in **[Appendix A.3]**) are indicated by grey scale of the group centroids with white corresponding to the lowest (i.e., control) and black to the highest tested concentration, respectively. Stress values are provided as a measure of "goodness-of-fit" for NMDS, with reasonable fits indicated when below 0.2 (Clarke, 1993) **[Appendix A.3]**.

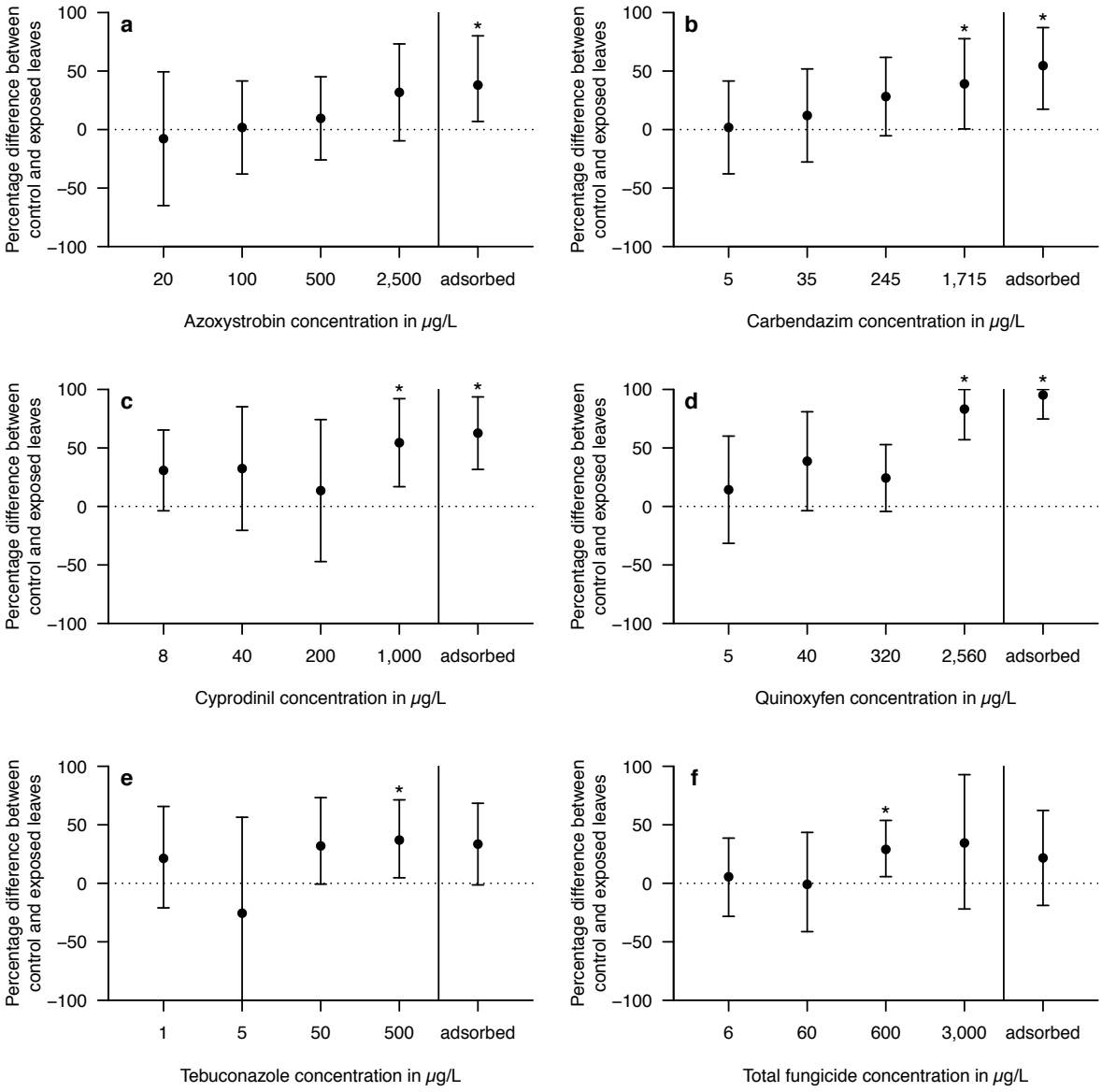

Fig. 4.6. Mean or median percentage difference (with 95% CI) in the feeding of *G. fossarum* on leaf discs conditioned in the presence of different concentrations of a) azoxystrobin, b) carbendazim, c) cyprodinil, d) quinoxyfen, e) tebuconazole, and f) the fungicide mixture compared to control discs. Positive effect sizes imply less feeding on the fungicide-exposed discs compared to the control and *vice versa*. Asterisks denote statistically significant differences compared to control leaf discs **[Appendix A.3]**.

4.3 IMPORTANCE OF WATERBORNE TOXICITY AND DIET-RELATED EFFECTS FOR SHREDDERS

Consistent with the observations during the second phase, the presence of 25 µg Cu/L during microbial conditioning significantly increased leaf-associated fungal biomass (~50%). Also, Cu-content of this leaf material was significantly higher (~200%) **[Appendix A.4]**. Consumption of these leaves by *G. fossarum* over 24 days triggered a significantly lower leaf consumption (~10%; Fig. 4.7) as well as a near-significant reduction in growth, a significant decrease in lipid content, and a significant increase in Cu-content of the gammarids (~30%, ~20%, and ~30%; Fig. 4.8). The latter was also observed for animals subjected to waterborne exposure towards 25 µg Cu/L (~50%; Fig. 4.8c). However, the only biological effect going along with this increased Cu-content in gammarids exposed via the water phase was a significant increase in feces production by ~10% (Fig. 4.7). The two effect pathways, namely waterborne toxicity and diet-related effects, resulted exclusively in additive effects (non-significant interaction terms of 2x2 ANOVAs) on all endpoints related to biological effects **[Appendix A.4]**.

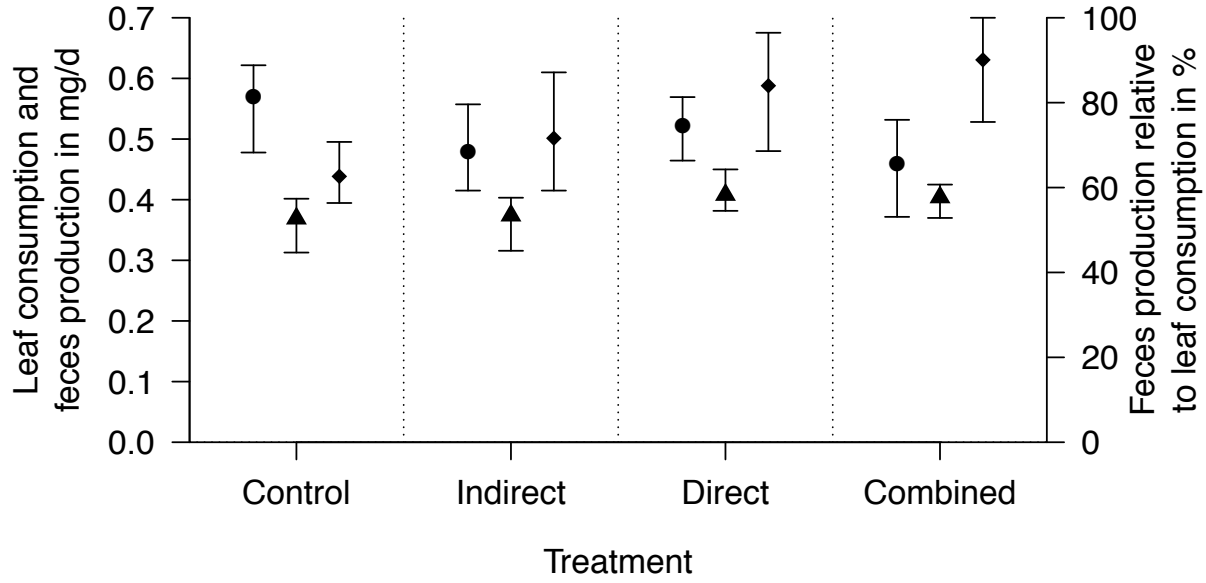

Fig. 4.7. Medians (with 95% CIs) of leaf consumption (points) and absolute (triangles) and relative (diamonds) feces production of *G. fossarum* being subjected for 24 days to four treatments: a Cu-free control, gammarids receiving leaves conditioned in the presence of Cu (i.e., indirect), gammarids being directly exposed to Cu (i.e., direct), and a combination of the indirect and direct treatments. For statistical evaluation see text **[Appendix A.4]**.

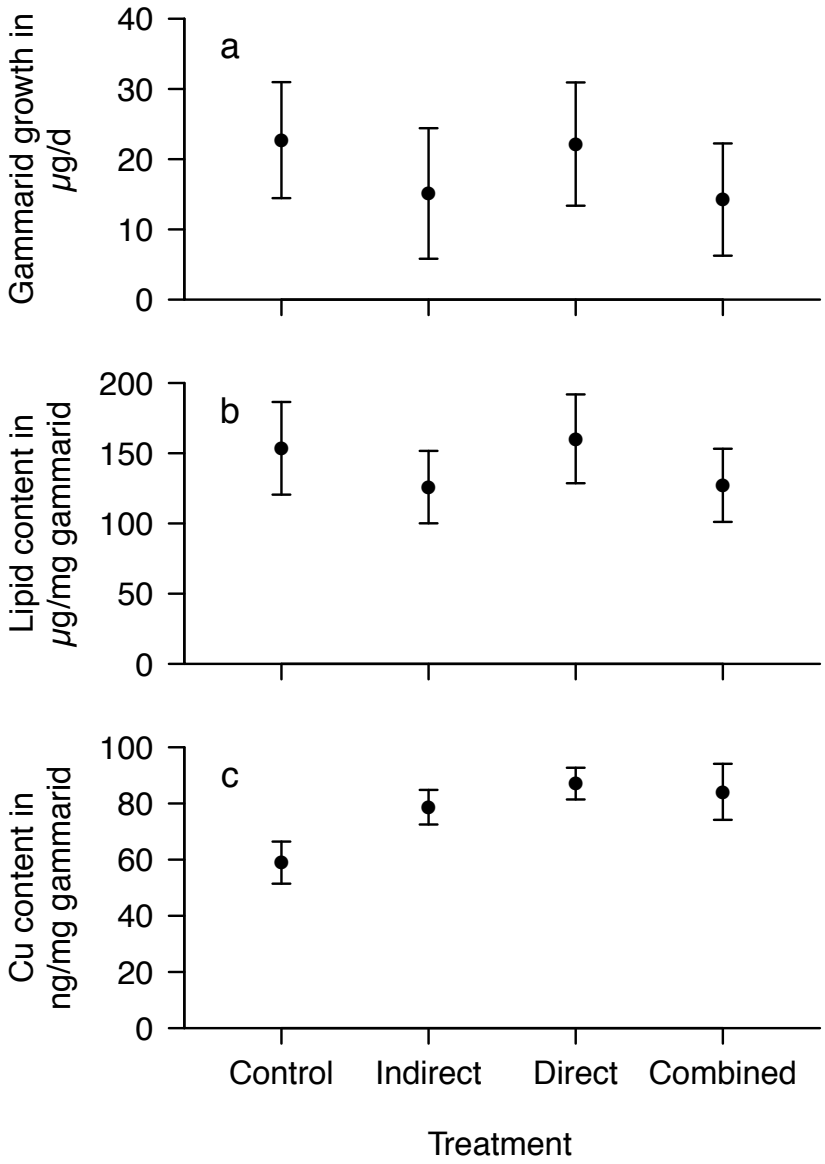

Fig. 4.8. Means (with 95% CIs) of (a) growth as well as (b) lipid and (c) Cu-content of *G. fossarum* being subjected for 24 days to four treatments: a Cu-free control, gammarids receiving leaves conditioned in the presence of Cu (i.e., indirect), gammarids being directly exposed to Cu (i.e., direct), and a combination of the indirect and direct treatments. For statistical evaluation see text **[Appendix A.4]**.

The presence of the organic fungicide mixture (62.5 µg/L sum concentration) during microbial conditioning did not significantly alter leaf-associated fungal biomass but significantly reduced fungal species richness (~40%) **[Appendix A.5]**. Consumption of such leaf material resulted in a significantly lower feces production by *G. fossarum* (~10%; Fig. 4.9) as did waterborne exposure towards the fungicide mixture (~30%), which also triggered an inhibited leaf consumption (~20%; Fig. 4.9). Both effect pathways drastically reduced gammarid growth (~110% and ~40% for waterborne toxicity and diet-related effects, respectively; Fig. 4.10), while strictly resulting in

additive effects (non-significant interaction terms of 2x2 ANOVAs) for all endpoints **[Appendix A.5]**.

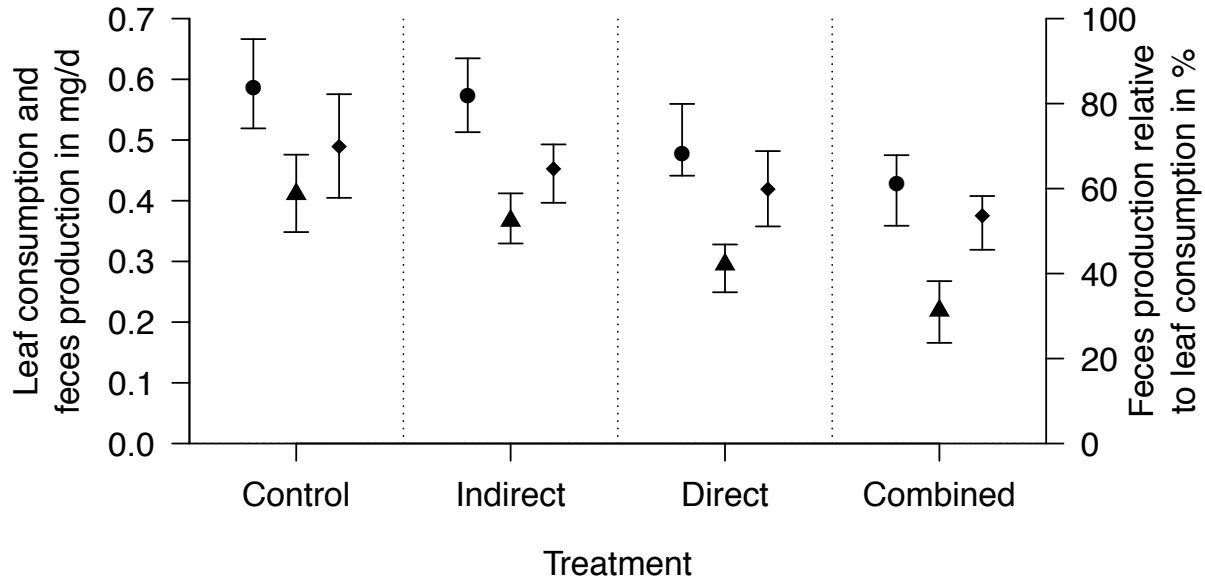

Fig. 4.9. Medians (with 95% CIs) of leaf consumption (points) and absolute (triangles) and relative (diamonds) feces production of *G. fossarum* being subjected for 24 days to four treatments: a fungicide-free control, gammarids receiving leaves conditioned in the presence of fungicides (i.e., indirect), gammarids being directly exposed to fungicides (i.e., direct), and a combination of the indirect and direct treatments. For statistical evaluation see text **[Appendix A.5]**.

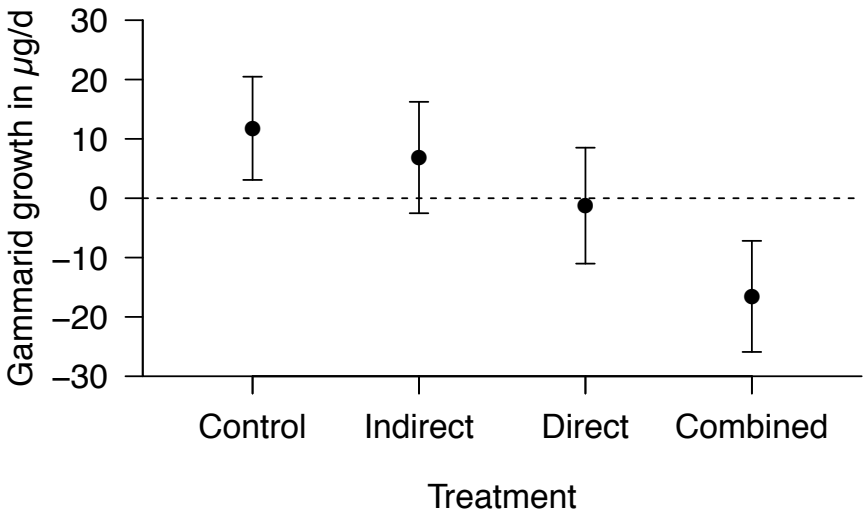

Fig. 4.10. Mean growth of *G. fossarum* (with 95% CIs) being subjected for 24 days to four treatments: a fungicide-free control, gammarids receiving leaves conditioned in the presence of fungicides (i.e., indirect), gammarids being directly exposed to fungicides (i.e., direct), and a combination of the indirect and direct treatments. For statistical evaluation see text **[Appendix A.5]**.

5. DISCUSSION

5.1 RESEARCH QUESTION 1: EFFECTS OF INORGANIC AND ORGANIC FUNGICIDES

In the course of this thesis, considerable differences were uncovered regarding the ecotoxicological potential of inorganic and organic fungicides in the model decomposer-detritivore system: while, during the first experimental phase, for all organic fungicides sublethal effects on gammarids' feeding activity as a result of waterborne exposure could be demonstrated using a static test design, this was not possible for inorganic fungicides **[Appendix A.1]**. For Cu, this was likely related to its fast dissipation from the water phase due to being highly adsorptive (FOOTPRINT, 2015), allowing for compensatory feeding behavior during later stages of the experiments **[Appendix A.1]**. By employing a semi-static test design, the expected sublethal effects (cf. Tattersfield, 1993) could be demonstrated for Cu (Table 4.1). However, using this approach, S still did not result in any sublethal or lethal effects in *Gammarus* **[Appendix A.1]**, supporting the general assumption of this fungicide being non-toxic to aquatic animals (Williams and Cooper, 2004).

But, during the second phase of this thesis, it was observed that leaf palatability was also not affected when microbial conditioning took place in the presence of S (Fig. 4.4). In contrast, organic fungicides affected leaf palatability negatively (cf. Bundschuh et al., 2011), which was displayed by a preference of *Gammarus* for control over fungicide-exposed leaves (Fig. 4.6), while for Cu the opposite was observed (Fig. 4.4). The preference for Cu-exposed leaf material may be caused by the positive effect of Cu on leaf-associated fungal biomass (Fig. 4.3), the most nutritious component of decomposing leaf material for most shredders (as reviewed by Suberkropp, 1992). Since fungi and bacteria can act antagonistically on leaf material (Gulis and Suberkropp, 2003), the observed general trend to lower bacterial densities under Cu-exposure (Fig. 4.3) may have reduced the competitive pressure for fungi resulting in higher fungal biomasses **[Appendix A.2]**. Another plausible explanation for the preference for Cu-exposed leaves may be the ability of *Gammarus* spp. to discriminate between different fungal species (e.g., Arsuffi and Suberkropp, 1989; Rong et al., 1995). Accordingly, the observed Cu-induced shift in aquatic hyphomycete community composition **[Appendix A.2]** potentially triggered the altered leaf palatability for *Gammarus* (cf. Bundschuh et al., 2011) but also the Cu-induced reduction in microbial leaf decomposition. The latter may be assumed since aquatic hyphomycete species decompose leaves at different rates (Duarte et

al., 2006) and Cu-exposure may have favored less effective decomposers (note that no explanation related to microbial community structure could be found for the S-induced reduction in microbial leaf decomposition **[Appendix A.2]**). Similarly, organic fungicide exposure affected aquatic hyphomycete community composition (Fig. 4.5) potentially triggering the responses observed for microbial leaf decomposition **[Appendix A.3]** as well as the negative effects on leaf palatability for the model shredder (Fig. 4.6). However, there was no obvious microorganism-related explanation for the food-choice response induced by quinoxyfen (Fig. 4.5) **[Appendix A.3]** and the effect sizes observed during the main and the adsorption experiments, which excluded any fungicide-induced changes in the leaf-associated microbial communities, were in good agreement for all fungicides (Fig. 4.6). This indicates a repellent effect caused by the fungicides adsorbed to the leaf material as the most likely explanation for the feeding patterns observed for organic fungicides (cf. Hahn and Schulz, 2007; Rasmussen et al., 2012).

Although an adsorption-related explanation for the preference of gammarids for leaf material exposed to Cu is unlikely (Tattersfield, 1993), during the third phase, Cu was nonetheless shown to accumulate on leaf material when present during conditioning **[Appendix A.4]**. The subsequent dietary uptake of Cu by *Gammarus*, during the 24-day long bioassay, seems to be the likely trigger for the reduced leaf consumption (Fig. 4.7) and physiological fitness (Fig. 4.8), even overriding the presumably positive nutritional effect related to the increased leaf-associated fungal biomass **[Appendix A.4]**. However, while the Cu-content of gammarids feeding on exposed leaf material was even exceeded by animals subjected to waterborne exposure (Fig. 4.8c), the latter did not cause any of the ecotoxicological effects observed for the diet-related path (Figs. 4.7 and 4.8). A potential reason for the absence of ecotoxicological implications in those organisms may be that the mechanisms available in crustaceans for protection against Cu-toxicity, namely sequestration in the hepatopancreas as non-bioavailable complexes and excretion with the feces (as reviewed by Schaller et al., 2011), may be more effective for waterborne exposure. The significant increase in absolute feces production (Fig. 4.7) due to the waterborne effect pathway, which was not observed for the diet-related path, could be interpreted as support for this explanation **[Appendix A.4]**. In stark contrast, the mixture composed of all organic model fungicides triggered a reduced feces production via both effect pathways (Fig. 4.9) **[Appendix A.5]**. For the waterborne effect path this may point to a compensational mechanism to cover the reduced energy uptake via

feeding (Fig. 4.9) but also an increased energy demand due to toxic stress (Maltby, 1999). Likewise, for the diet-related pathway, the reduction in feces production may be related to compensation of toxic effects due to the co-ingestion of fungicides adsorbed to the leaf substrate. In addition, exposure to organic fungicides during conditioning reduced fungal species richness associated with leaf material and induced a shift in the fungal community that *inter alia* increased the contribution of *Heliscus lugdunensis* SACCARDO & THERRY and *Tetracladium marchalianum* DE WILDEMAN, two species rejected by gammarids (Arsuffi and Suberkropp, 1989; Gonçalves et al., 2014), to total fungal sporulation **[Appendix A.5]**. Presuming that an active rejection of a fungal species indicates a lower food quality for a shredder, the reduced feces production in response to the diet-related effect pathway may thus also be interpreted as compensation for a fungicide-induced reduction in food quality. However, irrespective of the effect pathway and the actual underlying effect mechanism, compensation was seemingly incomplete as both paths resulted in meaningful reductions in gammarid growth (Fig. 4.10) **[Appendix A.5]**.

Thus, during all phases of this thesis, considerable differences regarding the effects of inorganic and organic fungicides in a decomposer-detritivore system were uncovered **[Appendix A.1-A.5]**. These observations in turn may indicate differing implications arising from fungicide use in organic (solely inorganic fungicides) and conventional (both groups) farming. However, it must be stressed that – apart from their differences – both groups were shown to have the potential to almost exclusively reduce microbial leaf decomposition as well as leaf-feeding by the model shredder and thus to consistently negatively affect decomposer-detritivore systems at the functional level **[Appendix A.1-A.5]**. This may at least partly explain the reported lack of deviations in leaf litter breakdown rates between streams influenced by organic and conventional farming (Magbanua et al., 2010).

5.2 RESEARCH QUESTION 2: FUNGICIDES' MIXTURE TOXICITY

During this thesis, it was indeed observed that fungicides can interact. First, as the addition of sulfur did not change the total Cu-concentration in comparison to the respective single-substance Cu-bioassay **[Appendix A.1]**, the substantially reduced toxicity of Cu in the presence of S during the inorganic mixture assay of the first experimental phase of this thesis (Table 4.1; Fig. 4.1) seems to be due to physiological processes within the test organisms. Accordingly, studies in nutritional science uncovered that S can act as dietary antagonist of Cu in animals *inter alia* due to metabolization into sulfides that make Cu physiologically unavailable (e.g., Mills, 1985).

Similarly, S did not have a significant effect on leaf-associated microorganisms and the functions they provide when applied singly at 0.2 mg/L. However, the presence of this S-concentration resulted in significantly lower Cu-induced effect sizes for nearly all assessed endpoints or even caused reversed effect directions compared to the respective single-substance experiment with Cu (Figs. 4.3 and 4.4) **[Appendix A.2]**. Presumably, this antagonistic mixture effect of Cu and S in leaf-associated microbial communities is also related to altered metabolic processes such as sulfide formation (e.g., Mills, 1985) or the promotion of the synthesis of thiol-containing compounds, which are involved in the Cu-detoxification of aquatic hyphomycetes (Guimarães-Soares et al., 2007), due to the higher S-availability.

Likewise, when leaf-associated microorganisms were exposed to the organic fungicide mixture, a large fraction of the joint structural and functional effects was lower than predicted (Fig. 4.6) **[Appendix A.3]**. A plausible explanation for this may be a positive nutritional effect of quinoxyfen for aquatic hyphomycetes, which is indicated by a significantly increased leaf-associated fungal biomass when this fungicide was assessed singly **[Appendix A.3]**. This may be related to the exceptional enzyme inventory of these fungi allowing them to mineralize organic xenobiotics and utilize the stored energy (Krauss et al., 2011). As a consequence, aquatic hyphomycetes may have been capable of mitigating the negative effects of the remaining mixture components to some extent, for instance via energetic investments in detoxification (Solé et al., 2012). However, conclusions about the mixture experiments involving leaf-associated microorganisms need to be treated with caution since microbial inocula for the experiments were sampled during different seasons. Therefore, communities potentially varying in their sensitivity to

fungicide stress were used and tested at a point in time not necessarily representing their most sensitive state, which might affect interpretation **[Appendix A.2 and A.3]**.

In stark contrast to the above-discussed antagonisms, at high concentrations, the organic fungicide mixture resulted in a synergistic effect on gammarids' feeding activity (Fig. 4.2b) **[Appendix A.1]**, which was most likely caused by the presence of the azole fungicide tebuconazole in the mixture. Compounds of this fungicide class are synergists, for instance for azoxystrobin, probably due to effects on P450 monooxygenase enzymes that are involved in detoxification processes (Cedergreen et al., 2006).

Thus, while the present thesis was the first to assess the mixture toxicity of fungicides on organisms and interactions among the trophic levels involved in leaf litter breakdown (but see for mixtures of metals or different pesticide groups e.g., Duarte et al., 2008; Rasmussen et al., 2012), non-additive action was observed during all mixture experiments **[Appendix A.1-A.3]**. This renders mixture effects of fungicides hardly predictable and in turn complicates the interpretation of field experiments (Fernández et al., 2015).

5.3 RESEARCH QUESTION 3: IMPORTANCE OF WATERBORNE AND DIET-RELATED EFFECTS

In contrast to the common assumption that waterborne exposure to contaminants is the major effect path for detritus feeders (Abel and Bärlocher, 1988; Forrow and Maltby, 2000; Sridhar et al., 2001), it was demonstrated during the present thesis that for Cu the diet-related pathway may be a more important route: while waterborne exposure solely resulted in a slight increase in feces production (Fig. 4.7), the diet-related path reduced food uptake (Fig. 4.7) and triggered detrimental effects on gammarids' physiological fitness (Fig. 4.8) **[Appendix A.4]**. In contrast, for organic fungicides the effect path related to waterborne exposure caused stronger effects than the diet-related path (judged by effect sizes, Figs. 4.9 and 4.10) **[Appendix A.5]**, while the general applicability of this phenomenon to compounds of this fungicide group may be concentration-dependent: since aquatic hyphomycetes' sensitivity towards most fungicides appears to be higher than that of *Gammarus* **[Appendix A.1 and A.3]**, an opposite pattern may be observed at organic fungicide concentrations that are below sublethal effect thresholds for the waterborne effect pathway. In the field, this latter scenario seems more likely as the prophylactic use of fungicides (with up to ten applications per season) creates the potential for long-term exposures towards these substances but at concentrations below those assessed in the organic fungicide experiment of the third phase (e.g., Battaglin et al., 2010; Reilly et al., 2012), reducing the risk for waterborne toxicity at the shredder-level **[Appendix A.1]**. In contrast, detrimental effects on fungal species richness can be observed at fungicide concentrations in the low µg/L range **[Appendix A.3]**, which directs more attention towards the diet-related effect pathway. However, even at concentrations resulting in sublethal toxicity in shredders via waterborne exposure such as that applied during this thesis, diet-related effects can contribute considerably to the combined effect of organic fungicides (Fig. 4.9 and 4.10), which is due to the additive action of both effect paths **[Appendix A.5]**. This was also observed for Cu **[Appendix A.4]** and in turn indicates that neither pathway must be ignored when evaluating fungicide effects in shredders since the combined action of both paths represents the realistic worst case for these organisms (cf. Zubrod et al., 2011).

5.4 RESEARCH QUESTION 4: LEVEL OF PROTECTION GRANTED BY THE EUROPEAN ERA AND ENVIRONMENTAL RELEVANCE

The European Union's ERA of fungicides is a tiered approach, whose first step involves a standard set of acute and chronic toxicity tests (EFSA, 2013a). When comparing the ecotoxicological benchmarks from such standard tests with threshold concentrations established during this thesis (EC_{20}-values for the first and NOECs, i.e., no-observed effect concentrations, for the second and third phase), gammarids' and – less surprising – aquatic hyphomycetes' sensitivity towards all tested fungicides was comparable to or even higher than that of the most sensitive standard test organism (Table 5.1) **[Appendix A.1-A.5]**.

During first-tier ERA, safety factors of ten (for chronic experiments) or 100 (for acute experiments) are applied to the ecotoxicological benchmarks and the resulting regulatory acceptable concentrations (RACs) are finally compared to the predicted environmental concentration (EFSA, 2013a). Indeed, RACs, determined during the first tier, appear to provide a sufficient level of protection for the majority of effects observed during single-substance experiments in this thesis (Table 5.1). The only exception to this was tebuconazole, causing a significant effect on microbial leaf decomposition already at a concentration of 1 µg/L **[Appendix A.3]**. This concentration equals the RAC and further supports the high sensitivity of aquatic fungi towards demethylation-inhibiting fungicides (Artigas et al., 2012; Bundschuh et al., 2011; Dijksterhuis et al., 2011; Dimitrov et al., 2014; Flores et al., 2014; Zubrod et al., 2011), a group accounting for approximately 20% of all organic fungicides approved in the European Union **[Appendix A.3]**.

Moreover, if a risk was indicated during the first tier, one option for a refined ERA is higher-tier effect assessment (EFSA, 2013a). This procedure, which was used to assess the risk of another 20% of the organic fungicides (excluding demethylation-inhibiting fungicides) – but also of Cu (EFSA, 2013b) – approved in the European Union, results in a median increase in RACs by a factor of approximately ten compared to the first tier **[Appendix A.3]**. When applying this factor (i.e., multiplying all first-tier RACs by ten), all RACs of the tested organic fungicides (with the exception of carbendazim) exceeded the corresponding NOECs established during the second phase of this thesis and, in the case of quinoxyfen, the EC_{20} for gammarids' feeding activity as well (Table 5.1). Therefore, these analyses also suggest ecotoxicological effects in decomposer-detritivore systems at concentrations that are supposed to protect populations of aquatic organisms (i.e., RACs; EFSA,

2013a). This, in turn, indicates that the European Union's current ERA practices for individual organic fungicides may provide insufficient protection for decomposer-detritivore systems for up to 40% of the approved substances (i.e., demethylation-inhibiting fungicides and substances assessed via higher-tier effect assessment).

However, fungicides are barely detected as individual substances in the field (e.g., Bereswill et al., 2012; Reilly et al., 2012) and the above-described situation may be even worse for organic fungicide mixtures considering significant effects in leaf-associated microbial communities at a sum concentration of only 6 µg/L **[Appendix A.3]**. At this sum concentration, the concentrations of each individual component approximated the respective first-tier RAC (with the exception of carbendazim; Table 5.1), questioning the protection granted by these values for fungicide mixtures in general (cf. Schäfer et al., 2012). Consequently, this also applies to those RACs derived by the less conservative higher-tier approaches, which additionally do not appear to be protective for the toxicity endpoints established during the mixture experiments of the first and third phase (with the exception of tebuconazole). Moreover, considering the high organic fungicide concentrations that can be detected in the field (Table 5.1), with sum concentrations of up to 80 µg/L (Bereswill et al., 2012), the effect concentrations established for organic fungicide mixtures during the present thesis (Table 5.1) appear to be field-relevant (even without considering a safety factor as routinely applied in risk assesment contexts; e.g., Feckler et al., 2015).

This latter point is also true for Cu, whose ERA seems highly inappropriate to protect aquatic organisms in general: despite the very low RAC (Table 5.1), which would require field Cu-concentrations below the world median concentration of 3 µg/L in uncontaminated freshwater systems (Bowen, 1979), concentrations from tens (entry via run-off; Bereswill et al., 2012; 2013) to hundreds of micrograms per liter (entry via spray drift; Süss et al., 2006) can be detected in surface waters. While Cu-concentrations in the former range already may trigger structural and functional implications in leaf-associated microbial communities and result in lethal and sublethal effects on the model shredder (Table 5.1), concentrations in the latter range may result in extirpation of, amongst others, *G. fossarum* (Table 4.1) **[Appendix A.1]**.

But also S, whose introduction in aquatic ecosystems has not been assessed in the past despite its high authorized application rates (e.g., up to more than 50 kg/ha/a in hop production in Germany; BVL, 2015b), may be of concern: when ignoring the

Table 5.1. Toxicity endpoints (extracted from the Pesticide Properties DataBase; FOOTPRINT, 2015) from standard toxicity tests with the resulting RACs (endpoints used for calculation are printed in bold) for the assessed substances. In addition, toxicity endpoints from the three phases of this thesis are provided **[Appendix A.1-A5]**. All concentrations are given in µg/L or µg Cu/L.

Fungicide	Acute standard tests		Chronic standard tests				RAC	Maximum surface water concentration	Phase 1		Phase 2		Phase 3
	Daphnia	Fish	Algae	*Chironomus*	*Daphnia*	Fish			Single-substance EC_{20}	Mixture EC_{20}	Single-substance NOEC	Mixture NOEC	NOEC
Cu[a]	24.7	**11.1**	5.9	n.r.	19.5	n.r.	0.11	263[c]	36.4	48.8	<10	<10	<25
S[b]	>63	>63	>63	n.r.	n.r.	n.r.	n.c.	n.r.	-	-	200	-	-
Azoxystrobin	**230**	470	360	800	44	147	2.3	29.7[d]	79.0	12.6	<20	<1	<15
Carbendazim	150	190	>7,700	13.3	**1.5**	3.2	0.15	2.5[e]	32.5	10.4	5	<1	<7.5
Cyprodinil	220	2,410	2,600	240	**8.8**	83	0.88	2.2[c]	15.7	7.0	8	<1	<7.5
Quinoxyfen	**80**	270	27	128	28	14	0.8	0.02[e]	6.0	3.3	<5	<2	<7.5
Tebuconazole	2,790	4,400	1,960	2,510	**10**	12	1	9.11[d]	91.7	27.4	<1	<1	<25

[a] Values for Cu stem from Cu-hydroxide for standard organisms and Phase 2 and from Cu-sulfate for Phases 1 and 3, respectively
[b] All standard toxicity values reported as greater as water solubility
[c] Süss et al. (2006)
[d] Berenzen et al. (2005)
[e] LUWG (2011)
n.c. not calculable
n.r. not reported

maximum solubility of S in water, which seems reasonable because naturally occurring solvents can increase water solubility of plant protection products considerably (Chiou et al., 1986), the maximum predicted environmental concentration approximates the NOEC for microbial leaf decomposition (the only endpoint found to be affected by S; Table 5.1) **[Appendix A.2]**.

The results of this thesis therefore suggest that both inorganic and organic fungicides can trigger detrimental effects in decomposer-detritivore systems at field-relevant levels and that the European Union's current fungicide ERA may provide insufficient protection for these organism complexes for a large share of approved substances **[Appendix A.1-A.5]**. The development of reasonable refinements of ERA practices seems thus mandatory to safeguard the ecological integrity of decomposer-detritivore systems and the functions they provide.

6. CONCLUSION AND OUTLOOK

As intended, this thesis provides the first thorough assessment of the ecotoxicological implications of inorganic and organic fungicides in a decomposer-detritivore system and contributes to answering the four research questions raised. More specifically, the results of this thesis suggest that environmentally relevant concentrations of both inorganic and organic fungicides, that may result from routine application in conventional but also organic farming (cf. Süss et al., 2006), can result in detrimental effects in these organism complexes **[Appendix A.1-A.5]**. This indicates far-reaching consequences for the energy flow in the detritus-based food web due to its bottom-up regulation (Wallace and Eggert, 1997): lower rates of leaf breakdown by microbial decomposers and shredders as induced by both fungicide groups (Table 4.1; Figs. 4.7 and 4.9) **[Appendix A.1-A.5]**, for instance, may result in less leaf-bound energy becoming available to the stream food web or may slow down this integration process (cf. Maltby et al., 2002). Moreover, shredders' feces represent a high-quality food resource in stream food webs (Bundschuh and McKie, in press; Cummins and Klug, 1979). A reduced feces production by *Gammarus* in response to both the waterborne and the diet-related effect pathway of organic fungicides (Fig. 4.9) **[Appendix A.5]** but also a potentially deteriorated nutritional value of feces as a result of Cu-exposure (for more details see **[Appendix A.4]**) may therefore suggest fungicide-induced effects on food availability and quality for collecting species. In addition, shredders such as gammarids represent a key prey for a variety of aquatic and terrestrial predators (MacNeil et al., 1999). Impairments in shredders' physiological fitness (body size and/or lipid content) triggered by waterborne exposure or diet-related effects of inorganic and organic fungicides (Figs. 4.8 and 4.10) **[Appendix A.4 and A5]** may thus ultimately affect higher trophic levels in detritus-based food webs (Wallace and Eggert, 1997).

From these findings, two initial recommendations for ERA of fungicides can be abstracted: first, considering both their exceptionally high sensitivity towards these substances (Table 5.1) **[Appendix A.2 and A.3]** and their crucial ecological role (e.g., Bärlocher, 1985; Taylor and Chauvet, 2014), microorganisms involved in leaf litter breakdown should be included in fungicide ERA testing schemes (cf. EFSA, 2013a). Given the insufficient current state of knowledge regarding the sensitivity of single microbial species and the biological interactions within leaf-associated microbial communities prone to chemical stress, the use of community-based test systems such as the one employed during the second phase of this thesis seems

necessary to ensure an adequate level of protection (cf. Brandt et al., 2015). In this context, microbial leaf decomposition as well as leaf-associated fungal biomass and community composition emerged as most sensitive endpoints **[Appendix A.2 and A.3]** and should therefore be the minimum set of parameters assessed. Second, in the European Union's aquatic ERA, diet-related effects of pesticides are currently exclusively assessed for fish if the pesticides exhibit very high octanol/water partition coefficients (EFSA, 2013a). While the fungicides assessed during the present thesis do not fulfil this criterion (FOOTPRINT, 2015), the observed effects nonetheless point to the fact that these substances may critically affect ecosystem functioning at the lower trophic level of shredders via this effect path **[Appendix A.4 and A.5]**. In order to adequately assess the overall risk fungicides pose for decomposer-detritivore systems, the inclusion of long-term feeding studies involving detritus feeders in ERA testing schemes seems thus a sensible first step.

Moreover, several knowledge gaps can be identified whose filling are a prerequisite for a better understanding of fungicides' effect mechanisms as well as their environmental relevance and thus for the development of further reasonable refinements for the ERA of these substances. To name the most important: a high priority should be assigned to a systematic assessment of the adsorption dynamics of fungicides (but also other chemical stressors; e.g., Bundschuh et al., 2013) onto leaf material and the resulting toxicity triggered by its ingestion. Combining these efforts with a more detailed understanding of the development and (functional) composition of leaf-associated microbial communities by involving for instance molecular biological tools (Fernandes et al., 2011) would help to unravel the significance of both factors likely driving the quality of leaves for shredders. Another important point would be to clarify if the effects uncovered for the model shredder – known to be particularly sensitive towards a range of (chemical) stressors (e.g., Maltby et al., 2002) and to feature a highly selective food choice (Arsuffi and Suberkropp, 1989) – and microbial community are generalizable among the functional feeding group of shredders and microbial communities of different geographic origin, respectively. A hands-on approach to test this would be to repeat selected experiments of this thesis with (1) shredder species that are reported to be less sensitive and selective, such as the larvae of certain caddisfly species (Arsuffi and Suberkropp, 1989; Brix et al., 2001) together with a microbial community originating from the sample location of this thesis and (2) microbial communities originating from other sample locations that differ in characteristics such as climate

and geomorphology together with *G. fossarum*. Furthermore, efforts should be put in assessing fungicide effects under controlled laboratory conditions but in a more environmentally realistic context to facilitate a better understanding of field observations (Fernández et al., 2015). This would for instance involve assessing higher levels of biological organization (e.g., shredder populations), mirroring field-relevant exposure scenarios (such as long-term low-level exposures; Battaglin et al., 2010; Reilly et al., 2012), testing under conditions that are less optimal for decomposer-detritivore systems than those provided during the experiments of this thesis (e.g., regarding nutrient supply via medium; Gulis et al., 2006), and considering other (chemical) stressors potentially co-occurring with fungicides (Puccinelli, 2012).

Finally, in the future, the contamination of aquatic ecosystems with fungicides may locally even aggravate due to the expected intensification of fungicide application as a result of global climate change and invasive fungal pathogens (Hakala et al., 2011; Stokstad, 2004). Therefore, it will be all the more important to have a fungicide ERA at hand that provides protective estimates of fungicide field concentrations (but see Knäbel et al., 2013) and their effects on aquatic key organisms. Since the findings of this thesis suggest that the latter is presently not the case for decomposer-detritivore systems **[Appendix A.1-A.5]**, considering leaf-associated microbial communities as well as the diet-related effect path in detritus feeders in ERA testing schemes and filling the above-identified knowledge gaps seem to be the mandatory first steps to safeguard aquatic ecosystem functioning.

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APPENDIX

Manuscripts **[A.1-A.5]** represent the latest versions submitted to the respective journal.

APPENDIX A.1

Effects of current-use fungicides and their mixtures on the feeding and survival of the key shredder *Gammarus fossarum*

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ABSTRACT

Fungicides are frequently applied in agriculture and are subsequently detected in surface waters in total concentrations of up to several tens of micrograms per liter. These concentrations imply potential effects on aquatic communities and fundamental ecosystem functions such as leaf litter breakdown. In this context, the present study investigates sublethal and lethal effects of organic (azoxystrobin, carbendazim, cyprodinil, quinoxyfen, and tebuconazole) and inorganic (three copper (Cu)-based substances and sulfur) current-use fungicides and their mixtures on the key leaf-shredding invertebrate *Gammarus fossarum*. The feeding activity of fungicide-exposed gammarids was quantified as sublethal endpoint using a static (organic fungicides; 7 d test duration) or a semi-static (inorganic fungicides; 6 d test duration with a water exchange after 3 d) approach (n = 30). EC_{50}-values of organic fungicides were generally observed at concentrations resulting in less than 20% mortality, with the exception of carbendazim. With regard to feeding, quinoxyfen was the most toxic organic fungicide, followed by cyprodinil, carbendazim, azoxystrobin, and tebuconazole. Although all tested organic fungicides have dissimilar (intended) modes of action, a mixture experiment revealed a synergistic effect on gammarids' feeding at high concentrations when using "independent action" as the reference model (~35% deviation between predicted and observed effect). This may be explained by the presence of a synergizing azole fungicide (i.e. tebuconazole) in this mixture. Furthermore, lethal concentrations of all Cu-based fungicides assessed in this study were comparable amongst one another. However, they differed markedly in their effective concentrations when using feeding activity as the endpoint, with Cu-sulfate being most toxic, followed by Cu-hydroxide and Cu-octanoate. In contrast, sulfur neither affected survival nor the feeding activity of gammarids (up to ~5 mg/L) but reduced Cu-sulfate's toxicity when applied in a binary mixture. Sulfur-related metabolic processes which reduce the physiological availability of Cu may explain this antagonistic effect. For both fungicide mixtures, the present study thus uncovered deviations from the appropriate reference model, while ecotoxicological effects were observed at field relevant (total) fungicide concentrations. Additionally, for more than half of the tested single substances, a potential risk for *Gammarus* and thus for the ecological function mediated by these organisms was evident at concentrations measured in agriculturally influenced surface waters. These results suggest that risks to the fundamental ecosystem function of leaf litter breakdown

posed by fungicides may not be adequately considered during the regulation of these compounds, which makes further experimental efforts necessary.

KEYWORDS

antimicrobial – environmental risk assessment – invertebrate – leaf litter decomposition – mixture toxicity – organic farming

INTRODUCTION

Organic and inorganic (e.g. copper (Cu)) fungicides are used to control a vast number of fungal pathogens during crop production in conventional farming (Ivic, 2010). But also for use under organic farming regimes, inorganic fungicides (particularly Cu-based substances and sulfur) are authorized in large parts of the world, including the U.S. (United States Department of Agriculture, 2013) and many European countries (e.g. Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, 2013). Due to their frequent application, as they can for instance account for more than 90% of pesticide applications in vineyards (Roßberg, 2009), these substances can be regularly detected as mixtures in total concentrations of up to several tens of micrograms per liter (e.g. Berenzen et al., 2005; Bereswill et al., 2012; 2013). As a result, fungicide exposure may contribute to ecologically meaningful changes in aquatic communities and ecosystem functions such as leaf litter breakdown in the field (Schäfer et al., 2012).

In streams, this breakdown process involves – besides leaf colonizing microorganisms – detritivorous macroinvertebrates (i.e. shredders; Hieber and Gessner, 2002), which make energy that is bound in allochthonous organic matter available for local and downstream communities (Cummins and Klug, 1979). There is, however, a fundamental lack of studies on the leaf breakdown via feeding of involved macroinvertebrates which are directly exposed to fungicides. In this context, exclusively the effects of Cu (e.g. Tattersfield, 1993) and the azole fungicide tebuconazole (Zubrod et al., 2010) on *Gammarus* (Crustacea, Amphipoda), a key shredder genus (Dangles et al., 2004; Englert et al., 2013), have been assessed. Also, it remains unknown how fungicides interact if these substances are present as mixtures in surface waters and whether these mixtures exhibit substantial ecotoxicological effects at field relevant levels.

Therefore, the objective of the present study was to assess direct, toxic effects of a set of current-use organic and inorganic fungicides, which were applied as single

substances as well as mixtures, to *G. fossarum*. The feeding activity of this test species is a sensitive, robust and ecologically relevant ecotoxicological endpoint (Maltby et al., 2002), and was used as sublethal response in this study. The model substances comprised five organic fungicides: azoxystrobin, carbendazim, cyprodinil, quinoxyfen, and tebuconazole. These fungicides were selected on the basis of their detection in natural surface water bodies (e.g. Süss et al., 2006; Bereswill et al., 2012; 2013). At the same time, they cover a cross-section of organic fungicides' modes of action applied on the field including mitochondrial respiration, mitosis and cell division, amino acid and protein synthesis, signal transduction, and sterol biosynthesis as target processes (Fungicide Resistance Action Committee, 2007). Four inorganic substances (Cu-hydroxide, Cu-octanoate, Cu-sulfate, and sulfur) were selected to represent the two primarily used fungicides of this group (i.e. Cu and sulfur; Eurostat, 2007) and to comprise Cu-based substances with varying chemical properties and toxicities. After testing all nine substances individually, the joint ecotoxicity of two fungicide mixtures – one comprising all organic fungicides and one binary mixture of Cu-sulfate and sulfur – was assessed and tested for compliance with the mixture reference model "independent action" (IA). This model assumes mixture components to have dissimilar modes of action (Bliss, 1939). The separate consideration of organic and inorganic fungicide mixtures allowed for an initial estimation of potential risks for the leaf litter breakdown mediated by *Gammarus* caused by substances which are currently also authorized for organic agriculture.

MATERIALS AND METHODS

CHEMICALS

Information about the five organic and four inorganic fungicides used in this study is provided in Table 1. All fungicides were applied as commercially available formulations, rendering the use of further solvents unnecessary, with the exception of carbendazim, which was applied as a chemical standard (97%; Sigma-Aldrich) since no product containing only this active ingredient was commercially available in Germany at the time the study was performed. In addition, Cu-sulfate was applied as a pentahydrate salt (>99%; Fluka) to allow a direct comparison with literature data (e.g. Tattersfield, 1993). All fungicides were diluted in a standard artificial medium (SAM-5S; Borgmann, 1996). Only carbendazim was first diluted in ethanol (≥99.5 %) and subsequently serially-diluted using SAM-5S. All treatments of bioassays with carbendazim (including the control) received the same amount of ethanol (0.5‰),

Table 1. Classification, origin, ecotoxicologically relevant information, and tested concentrations of the assessed fungicides.

Fungicide	Chemical class	Chemical family	Product applied	Supplier	Mode of action[a]	Tested concentrations (µg/L or µg Cu/L)		Maximum surface water concentration (µg/L or µg Cu/L)	K_{OC}	Chronic *Daphnia* NOEC (µg/L or µg Cu/L) [b]	Range of reported sublethal / lethal toxicity thresholds for crustaceans (µg/L or µg Cu/L) [c]
						Single substance bioassays	Mixture bioassays				
Azoxystrobin	Organic	Strobilurins	Ortiva	Syngenta Agro GmbH	Inhibition of mitochondrial respiration	11.3; 22.5; 45.0; 90.0; 180.0	14.5; 17.6; 27.0; 30.1; 33.2	29.7 [d]	589 [b]	44	0.026 to 259 / 9.5 to 277
Carbendazim		Benzimidazoles	-	-	Inhibition of mitosis and cell division	25.0; 40.0; 55.0; 70.0; 85.0	12.0; 14.6; 22.3; 24.8; 27.4	1.6 [e]	200-246 [g]	1.5	2.17 to >100,000 / 3.1 to >100,000
Cyprodinil		Anilino-pyrimidines	Chorus	Syngenta Agro GmbH	Inhibition of amino acid and protein synthesis	6.0; 15.6; 40.6; 105.5; 274.2	8.1; 9.8; 15.0; 16.7; 18.4	2.2 [e]	1,470 [h]	8.8	1.9 to 70 / 3.7 to 8,140
Quinoxyfen		Quinolines	Fortress 250	Dow AgroSciences GmbH	Perturbation of signal transduction	5.0; 9.0; 16.2; 29.2; 52.5	3.8; 4.6; 7.1; 7.9; 8.7	0.02 [f]	>9,995 [b]	28	0.91 to 47.2 / 3.03 to 91
Tebuconazole		Triazoles	Folicur	Bayer CropScience	Inhibition of sterol biosynthesis	50.0; 92.5; 171.1; 316.6; 585.7	31.6; 38.4; 58.7; 65.5; 72.3	9.11 [d]	991 [i]	10	35 to 230 / 35 to 4,000
Cu-hydroxide	Inorganic	Inorganica	Cuprozin flüssig	Spiess-Urania Chemicals	Multi-site	30; 55; 80; 105; 130	-	263 [e]	12,000 [b]	19.6	- / 651.4 to 19,007,677.3
Cu-octanoate		Inorganica	Cueva Pilzfrei	W. Neudorff GmbH KG	Multi-site	45; 70; 95; 120; 145	-	263 [e]	100,000 [j]	-	- / 0.18 to 2.2
Cu-sulfate		Inorganica	-	-	Multi-site	15; 40; 65; 90; 115	15; 40; 65; 90; 115	263 [e]	9,500 [b]	31.4	0.007 to 11104.6 / 0.04 to 257090.6
Sulfur		Inorganica	Kumulus WG	BASF SE	Multi-site	60; 180; 540; 1,620; 4,860	165	-	1,950 [b]	-	- / 736,000 to 3,850,000

[a] Fungicide Resistance Action Committee (2007)

[b] FOOTPRINT (2013)

[c] ECOTOX Database (United States Environmental Protection Agency, 2013); note that immobilisation was considered as lethal effect

[d] Berenzen et al. (2005)

[e] Süss et al. (2006)

[f] Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht Rheinland-Pfalz (2011)

[g] European Commission (2007)

[h] PAN Pesticides Database (2010)

[i] Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (2009)

[j] Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (2010)

which is non-toxic to gammarids even in chronic exposures (Watts et al., 2002). Directly following the fungicide-application and at the termination of the bioassays (for the inorganic fungicides at the time of the medium renewal; see section 2.4), triplicate 10 mL-samples of the control treatments and the treatments with the lowest and the highest tested fungicide concentration(s) were collected for chemical analysis. Organic fungicide samples were immediately frozen at -20°C, while Cu-samples were acidified with nitric acid (~1% of the final volume) and stored at 4°C until further use. As the test medium already contained high levels of sulfur potentially masking the added amounts, sulfur concentrations from fungicide application (e.g. 2% in the inorganic mixture experiment) were not verified by chemical analysis. Chemicals were purchased from Sigma-Aldrich or Roth unless otherwise specified.

PREPARATION OF LEAF DISCS

Leaf discs were prepared as described in Zubrod et al. (2010). Briefly, black alder (*Alnus glutinosa* (L.) Gaertn.) leaves from trees near Landau, Germany (49°11'N; 8°05'E), were collected shortly before leaf fall in October 2011 and stored frozen at -20°C until further processing. Using a cork borer, discs of 2 cm diameter were cut from the leaves while excluding the main vein. Leaf discs were subjected to microbial colonization (i.e. conditioning) for 10 d in nutrient medium (Dang et al., 2005) using leaf material previously exposed in a near-natural stream (Rodenbach, Germany, 49°33'N, 8°02'E) as inoculum. After conditioning, leaf discs were dried to a constant weight (~24 h at 60°C) and weighed to the nearest 0.01 mg. Approximately 48 h prior to the start of each bioassay, leaf discs were re-soaked in SAM-5S to prevent floating at the surface of the test medium during the experiments.

TEST ORGANISMS

At least 7 d prior to each experiment, *G. fossarum* were kick-sampled in the near-natural stream Hainbach near Landau, Germany (49°14'N; 8°03'E; genetic analyses revealed that the population inhabiting this sample site is exclusively composed of animals belonging to cryptic lineage B; Feckler et al., 2012). In the laboratory, gammarids were immediately subjected to a passive underwater separation technique dividing them into different size classes (Franke, 1977). Only adult males – identified by their position in precopula pairs – of approximately 6 to 8 mm body length (measured as the base of the first antenna to the base of the telson) which

were visually free of acanthocephalan parasites were used in order to reduce variability in feeding behavior during the bioassays (*cf*. Naylor et al., 1989; Pascoe et al., 1995). Throughout the acclimation phase (≥7 d) in the laboratory, animals were kept in aerated medium in a climate-controlled chamber at 20±1°C in total darkness, while they were fed *ad libitum* with pre-conditioned black alder leaves and were gradually adapted to SAM-5S.

BIOASSAY DESIGNS

Each bioassay was performed using six treatments including five different fungicide concentrations and a fungicide-free control (all nominal concentrations are provided in Table 1) aiming to obtain a complete concentration-response curve of the fungicide or the mixture in terms of the feeding rate of *Gammarus* (with the exception of the inorganic mixture experiment; see section 2.5). Each treatment was comprised of 30 replicates. Each replicate consisted of one gammarid in a glass beaker filled with 200 mL aerated SAM-5S containing the respective fungicide concentration(s) and two pre-weighed leaf discs. Additionally, five replicates per treatment which did not include gammarids accounted for microbial and abiotic leaf mass losses during the experiments. After 7 d, mortality of the test organisms was recorded and, from the replicates with surviving gammarids, animals as well as remaining leaf material were removed, dried to a constant weight (~24 h at 60°C) and weighed to the nearest 0.01 mg. Note that by discarding animals that died during the bioassays, the true impacts on the population feeding rates could be underestimated in the present study. Moreover, as argued by Bundschuh and Schulz (2011), the experimental duration of 7 d is too short to allow a considerable recolonization of dried leaf material by microorganisms, especially when no fresh inoculum is present. Thus, during the present study, indirect fungicide effects by an altered food-quality would be highly unlikely.

Since preliminary experiments showed that Cu-concentrations decreased considerably within 3 d, which may allow compensatory feeding in the remaining test duration (Zubrod, personal observation), a semi-static approach was used for all experiments with inorganic fungicides. Each glass beaker was equipped with a cylindrical cage (height: 8.0 cm, diameter: 5.5 cm) made of stainless steel (material number: 1.4301) 0.5 mm-mesh screen containing the gammarid and the two leaf discs (Zubrod et al., 2010). These cages allowed a careful transfer of the animals and leaf discs to new vessels containing fresh SAM-5S with the respective fungicide

concentration after 3 d. Bioassays using inorganic fungicides were terminated after 6 d following the procedure detailed above.

DESIGNS OF THE MIXTURE EXPERIMENTS

To assess the joint toxicity of a mixture containing all five organic fungicides (throughout the remainder of the manuscript referred to as the “organic mixture”), a “fixed ratio” design based on EC_{50}-values was used (Jonker et al., 2011). The total fungicide concentration was varied (five different fungicide concentrations and a fungicide-free control) to allow the observation of a complete concentration-response curve regarding the feeding rate of *Gammarus*. The observed toxicity was then compared to IA expectations. Initially, it was planned to also test the compliance with “concentration addition” (CA), a reference model which assumes the mixture's components to have the same modes of action (Loewe and Muischnek, 1926), although this assumption was not met by the mixture tested in the present study. Nonetheless, the CA predictions were either partly (for feeding) or completely (for mortality) prevented by characteristics of the data (see for details Supplementary Material and Fig. A1), rendering it impossible to refute or to confirm compliance with CA.

A binary inorganic mixture consisting of the Cu-fungicide with the highest sublethal toxicity (i.e. Cu-sulfate; see section 3.2) as well as sulfur was assessed (throughout the remainder of the manuscript referred to as the “inorganic mixture”). Since sulfur was non-toxic in the tested concentration range during the single substance assessment (see section 3.2), which also prevented a comparison of the joint toxicity with CA expectations, an “A in the presence of B” design (Jonker et al., 2011) was employed to test the joint toxicity against IA expectations. The Cu-sulfate concentrations already tested when applied singly were investigated in combination with a fixed sulfur concentration of 165 µg/L. This concentration was chosen on the basis of the maximum predicted environmental concentration derived from FOCUS exposure modeling (Forum for Co-ordination of Pesticide Fate Models and their Use, 2001), ignoring sulfur’s maximum solubility in water.

FUNGICIDE ANALYSIS AND PHYSICO-CHEMICAL PARAMETERS OF TEST MEDIUM

Organic fungicide and Cu-concentrations were measured using an ultra-HPLC-MS (Zubrod et al., 2011) and a quadrupole ICP-MS (Rosenfeldt et al., 2015), respectively

(both Thermo Fisher Scientific). Nominal and measured initial organic fungicide concentrations were in strong agreement with a maximum deviation of 12% and an average of 4%, indicating an accurate assessment of the fungicides' toxicity and the use of nominal concentrations throughout this paper (Table 2). Although blanks with high variation resulted in comparably high limits of detection and quantification for cyprodinil, we consider these measurements valid given the good agreement with the nominal concentrations. In addition, despite the involvement of a special ion exchanger, which was used to further reduce the load of ions in the deionized water from which SAM-5S was prepared, we did not achieve a control Cu-concentration below the limit of detection for all bioassays. However, our highest measured control Cu-concentration is still below the world median Cu-concentration of 3 µg/L in uncontaminated freshwater systems reported by Bowen (1979). Furthermore, the highest Cu-control-levels of the present study are comparable to a concentration successfully used as control in a 100 days-lasting population experiment using *Gammarus* (e.g. Maund et al., 1992). Therefore, our control treatments can be considered suitable for the present study.

Fungicide analyses were supplemented by the physico-chemical characterization of fresh, 3-d old (at the medium exchange during inorganic fungicide assays), and 7-d old (at the termination of organic fungicide assays) SAM-5S (n=3) using a WTW Multi 340i/SET as well as Macherey-Nagel visocolor® and nanocolor® kits. Results of these measurements are provided in Table A1.

CALCULATIONS AND STATISTICS

Feeding rates of gammarids were expressed as mg consumed leaf mass per mg dry weight of *Gammarus* per day and were calculated as described in Zubrod et al. (2010). To determine the fungicides' effective and lethal concentrations resulting in 20 or 50% inhibition of gammarids' feeding rate and mortality (i.e. EC_{20}- and EC_{50}-values as well as LC_{20}- and LC_{50}-values), respectively, several concentration-response models were fitted to each data set using the R extension package "drc" (Ritz and Streibig, 2005). The model fitting the data best was selected based on Akaike's information criterion as well as expert judgment (all models together with their parameters are provided in Table A2). Only EC-/LC-values falling within the range of the tested fungicide concentrations are reported in the present study. Using the calculation methods described in Altman et al. (2000), 95% confidence intervals (CIs) for group means or medians (if data was not normal, which was assessed using

Table 2. Nominal and measured (means with 95% confidence intervals (CIs)) fungicide concentrations in the bioassays together with the respective limits of detection (LODs) and limits of quantification (LOQs).

Fungicide	LOD (µg/L or µg Cu/L)	LOQ (µg/L or µg Cu/L)	Single substance bioassays				Mixture bioassays			
			Nominal (µg/L or µg Cu/L)	Measured at start (µg/L or µg Cu/L)	Measured at termination (µg/L or µg Cu/L)[a]	% present at termination[a]	Nominal (µg/L or µg Cu/L)	Measured at start (µg/L or µg Cu/L)	Measured at termination (µg/L or µg Cu/L)[a]	% present at termination[a]
Azoxystrobin	1.98	6.61	0.0	<LOD	<LOD	-	0.0	<LOD	<LOD	-
			11.3	11.29 (10.32-12.26)	9.99 (9.53-10.46)	88.5	14.5	15.28 (13.75-16.80)	12.64 (10.67-14.61)	82.7
			180.0	181.59 (146.83-216.34)	164.14 (131.28-197.00)	90.4	33.2	32.09 (26.91-37.26)	29.11 (26.17-32.06)	90.7
Carbendazim	0.53	1.75	0.0	<LOD	<LOD	-	0.0	<LOD	<LOD	-
			25.0	25.52 (21.86-29.18)	22.31 (21.14-23.49)	87.4	12.0	12.11 (11.65-12.58)	10.79 (9.63-11.94)	89.1
			85.0	84.85 (72.00-97.73)	79.34 (63.08-95.60)	93.5	27.4	26.47 (24.37-28.57)	24.22 (19.65-28.80)	91.5
Cyprodinil	3.91	13.02	0.0	<LOD	<LOD	-	0.0	<LOD	<LOD	-
			6.0	5.53 (4.40-6.65) (<LOQ)	3.76 (3.18-4.34) (<LOD)	68.0	8.1	7.25 (5.90-8.60) (<LOQ)	4.95 (3.21-6.69) (<LOQ)	68.3
			274.2	278.38 (211.29-345.48)	239.85 (188.80-290.90)	86.2	18.4	17.73 (15.40-20.05)	12.46 (9.66-15.26) (<LOQ)	70.3
Quinoxyfen	0.37	1.23	0.0	<LOD	<LOD	-	0.0	<LOD	<LOD	-
			5.0	5.04 (3.54-6.55)	1.28 (0.00-3.07)	25.4	3.8	3.45 (2.94-3.96)	1.41 (0.00-5.23)	40.9
			52.5	50.91 (24.99-76.86)	11.16 (10.95-11.37)	21.9	8.7	8.71 (6.83-10.59)	1.15 (0.73-1.58) (<LOQ)	13.2
Tebuconazole	2.11	7.05	0.0	<LOD	<LOD	-	0.0	<LOD	<LOD	-
			50.0	54.13 (39.75-68.52)	50.75 (44.38-57.12)	93.8	31.6	30.00 (25.95-34.05)	29.21 (23.58-34.84)	97.4
			585.7	569.60 (487.04-652.16)	540.69 (442.61-638.78)	94.9	72.3	74.20 (69.61-78.79)	68.22 (64.39-72.04)	91.9
Cu-hydroxide			0.0	1.30 (1.24-1.36)	0.81 (0.75-0.86)	62.3	-	-	-	-
			30.0	30.33 (12.48-48.19)	10.67 (8.61-12.72)	35.2	-	-	-	-
			130.0	128.70 (107.57-149.83)	82.89 (0.00-181.90)	64.4	-	-	-	-
Cu-octanoate			0.0	<LOQ	<LOQ	-	-	-	-	-
	0.23	0.72	45.0	43.19 (41.05-45.33)	8.95 (4.45-13.45)	20.7	-	-	-	-
			145.0	158.92 (150.18-167.66)	72.19 (32.91-111.47)	45.4	-	-	-	-
Cu-sulfate			0.0	<LOQ	<LOQ	-	0.0	2.44 (2.36-2.51)	1.19 (1.16-1.23)	48.8
			15.0	13.86 (13.52-14.19)	4.55 (3.98-5.12)	32.8	15.0	15.38 (14.85-15.90)	4.32 (2.74-5.90)	28.1
			115.0	112.97 (109.56-116.39)	65.83 (9.04-122.63)	58.3	115.0	116.21 (114.24-118.17)	47.80 (2.19-97.79)	41.1

[a] for organic fungicides after 7 d, for inorganic ones after 3 d

histograms and expert judgment) and proportions of dead animals were determined for use in graphics. Moreover, no-observed effect concentrations (NOECs) are – for the sake of completeness – provided in Table A3. It should, however, be noted that the underlying concept is frequently criticized (e.g. Bundschuh et al., 2013). One aspect in this context is that the statistical power to detect small effect sizes on the basis of null hypothesis significance testing may be low if the variability is relatively high or the replication not sufficient. Since this was partly the case in the present study, the provided NOECs should be interpreted with caution.

The expected joint effect of the two mixtures according to IA was formulated as (see e.g. Backhaus et al., 2000):

$$E(c_{mix}) = E(c_1 * c_2 * \ldots * c_N) = 1 - \prod_{i=1}^{n}[1 - E(c_i)] \quad (1)$$

where $E(c_{mix})$ is the predicted mixture effect scaled to the range 0 to 1, c_{mix} is the total fungicide concentration in the mixture, c_i is the concentration of the ith fungicide, and $E(c_i)$ the effect (also scaled to the range 0 to 1) of the ith fungicide applied singly at the given concentration. For the feeding data of the present study, $E(c_{mix})$ was thus calculated as:

$$E(c_{mix}) = 1 - P(c_1 * c_2 * \ldots * c_N) = 1 - \prod_{i=1}^{n}[P(c_i)] \quad (2)$$

where $P(c_i)$ denotes the proportion derived by dividing the feeding rate observed at c_i by the control feeding rate, with both rates being observed in the bioassay where the fungicide was applied singly (*cf*. Rasmussen et al., 2012). In addition, equation 1 was used to calculate IA predictions for mortality. R version 2.15.3 (www.r-project.org) for Mac was used for all statistics and figures. Note that the term “significant” is exclusively used in reference to statistical significance throughout this study.

RESULTS AND DISCUSSION

ORGANIC FUNGICIDES

EC_{50}-values of organic fungicides were generally observed at concentrations which caused less than 20% mortality, with the exception of carbendazim showing high lethal toxicity, with its LC_{50} being even lower than its EC_{50} (Table 3; Fig. 1). Cuppen et al. (2000) also found a high mortality in carbendazim-exposed *G. pulex* with a complete elimination of the gammarids at 100 µg/L matching well with our data. With regard to feeding, quinoxyfen was the most toxic organic fungicide, followed by cyprodinil, carbendazim, azoxystrobin, and tebuconazole (Table 3; Fig. 1). In general, the EC- and LC-values derived in the present study lie within the range of toxicity thresholds reported for crustaceans (Table 1). Using *G. pulex*, Beketov and Liess

(2008) also studied sublethal toxicity (i.e. drift) of azoxystrobin and cyprodinil. While the absence of an effect of azoxystrobin at 20 µg/L reported by these authors corroborates the present study's results, cyprodinil appears to affect feeding rate more effectively than drift, which may be triggered by differences in exposure duration. Zubrod et al. (2010) also assessed the implications of tebuconazole on the feeding rate of *G. fossarum*, resulting in a 7-day EC_{50} of approximately 300 µg/L. This value is approximately 50% higher than what was observed in the present study (Table 3), which is likely driven by the use of natural stream water as test medium in the study of Zubrod et al. (2010). Natural stream water can contain high amounts of dissolved organic carbon reducing the toxicity of *inter alia* organic pesticides (e.g. Day, 1991).

Table 3. EC- and LC-values derived from bioassays with single fungicides or mixtures. Concentrations are given in µg/L or µg Cu/L and are supplemented by 95% CIs.

Fungicide	EC_{20}	EC_{50}	LC_{20}	LC_{50}
Azoxystrobin	79.0 (30.2-127.7)	90.8 (79.1-102.5)	98.0 (70.4-125.6)	148.4 (127.8-169.1)
Carbendazim	32.5 (14.3-50.7)	75.0 (47.5-102.6)	35.3 (13.5-57.1)	51.0 (43.6-58.5)
Cyprodinil	15.7 (1.3-30.2)	50.5 (0.0-103.3)	73.9 (13.2-134.6)	-
Quinoxyfen	6.0 (0.1-12.1)	23.8 (11.5-36.1)	-	-
Tebuconazole	91.7 (38.6-144.8)	197.8 (144.1-251.6)	-	-
Cu-hydroxide	65.61 (0.0-426.7)	-	58.7 (48.1-69.4)	89.1 (81.3-96.9)
Cu-octanoate	85.2 (58.5-111.8)	123.2 (100.7-145.7)	60.0 (49.0-71.0)	91.1 (82.0-100.2)
Cu-sulfate	36.4 (21.7-50.0)	67.9 (46.5-89.4)	66.2 (62.0-70.4)	77.8 (73.6-81.9)
Sulfur	-	-	-	-
Organic mixture	60.6 (32.3-89.0)	119.5 (97.3-141.6)	-	-
Inorganic mixture	48.8 (30.0-67.6)	-	72.2 (61.7-82.7)	96.7 (90.6-102.8)

The organic fungicide mixture caused no statistically significant deviation from IA with regard to mortality (predictions included in 95% CIs of observations; Fig. 2a). This aligns with the expectations for a mixture composed of substances with dissimilar modes of action (Kortenkamp and Altenburger, 2011) as applied in the present study (Table 1). Moreover, the observed feeding rate of gammarids exposed to the organic mixture agreed well with the prediction by IA for the lowest tested total fungicide concentration (70 µg/L; Fig. 2b). At higher concentrations, however, observed feeding rates were lower than predicted by IA. The deviation was statistically significant only at the highest tested mixture concentration (160 µg/L) with the observed feeding rate inhibition compared to the control deviating by approximately 35% from that predicted by the reference model. However, a significant deviation

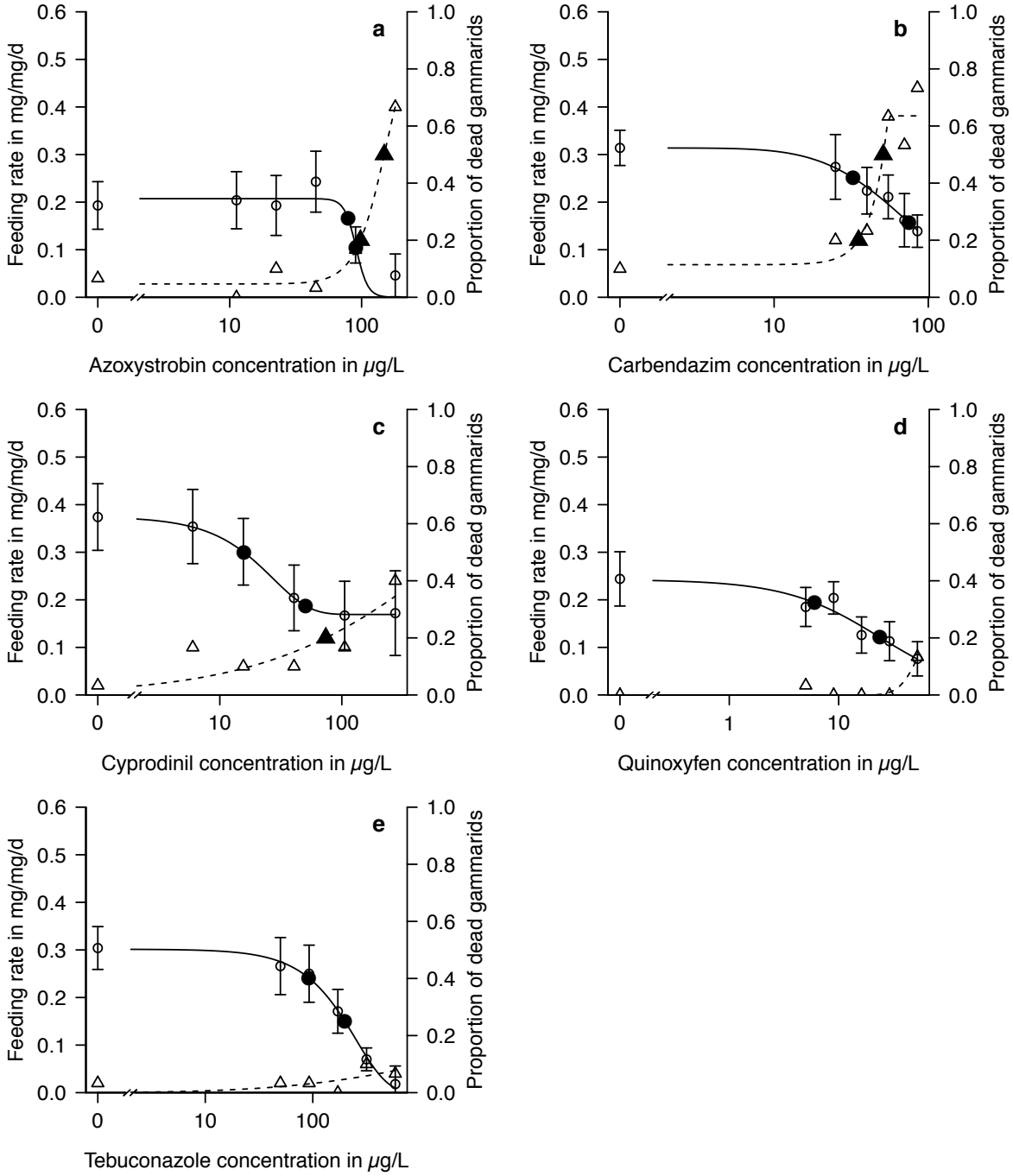

Fig. 1. Mean feeding rates (open circles) with 95% confidence intervals (CIs) of *G. fossarum* and proportion of dead animals (open triangles) when exposed to different concentrations of a) azoxystrobin, b) carbendazim, c) cyprodinil, d) quinoxyfen, and e) tebuconazole. The best fitting models for feeding rate (solid line) and mortality (dashed line) as well as EC- (solid circles) and LC-values (solid triangles) for 20 and 50% are also displayed.

was already observed at 135 µg/L during a range-finding experiment (also revealing a higher than expected effect with regard to mortality at very high concentrations; Fig. A2). This synergistic (i.e. higher than expected from the reference model) mixture effect suggests a joint interaction of the mixture's components. Although modifications of the mixture toxicity by solvents and additives cannot be ruled out (*cf*.

Adam et al., 2008), it appears more likely that the observed pattern is caused by the presence of the azole fungicide tebuconazole. Compounds of this fungicide class are synergists, for instance for azoxystrobin, probably due to effects on P450 monooxygenase enzymes that are involved in detoxification processes (Cedergreen et al., 2006), although no synergistic effect was found for tebuconazole when applied jointly with a pyrethroid insecticide (Nørgaard and Cedergreen, 2010).

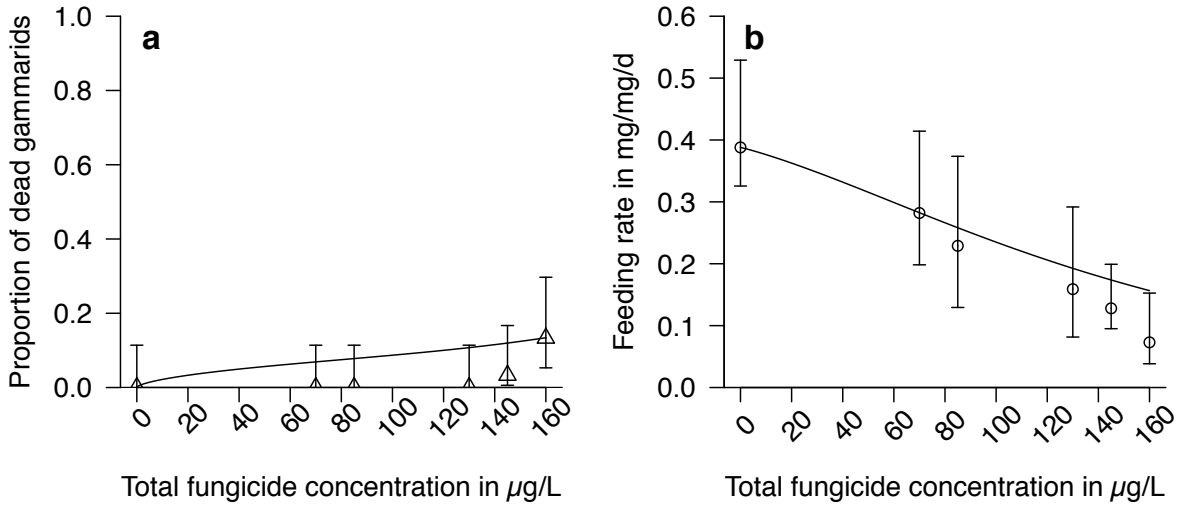

Fig. 2. Proportion of dead animals (triangles; a) and median feeding rates (circles; b) of *G. fossarum* – with 95% CIs – when exposed to different concentrations of the organic mixture. The solid lines indicate predictions by IA.

INORGANIC FUNGICIDES

Sulfur (up to almost 5 mg/L) was the only fungicide tested in the present study for which neither lethal nor sublethal effects on *G. fossarum* could be detected (Table 3; Fig. 3). These results support the general assumption of sulfur being non-toxic to aquatic animals (Williams and Cooper, 2004). The Cu-based fungicides, in contrast, caused high mortalities. All three substances were of comparable lethal toxicity with LC_{20}- and LC_{50}-values deviating only by approximately 13 and 17%, respectively (Table 3; Fig. 3). However, with regards to their sublethal toxicity (i.e. feeding inhibition), Cu-compounds differed considerably: While for Cu-sulfate the EC_x-values were lower than the corresponding LC_x-values (Table 3), which is in accordance with the observations of Tattersfield (1993) for *G. pulex*, the opposite observation was made for Cu-octanoate. Moreover, given the high mortality, it was not possible to determine an EC_{50} for Cu-hydroxide (Table 3). EC-values of Cu-compounds were arranged in the same order as their corresponding K_{OC}-values (Tables 1 and 3). This indicates that the bioavailability of Cu-based fungicides may have been influenced in

a substance-specific manner via adsorption to dissolved organic carbon and the present leaf material, which is supported by the reduced recovery of Cu from Cu-octanoate in the water phase after three days compared to both Cu-sulfate and Cu-hydroxide (Table 2).

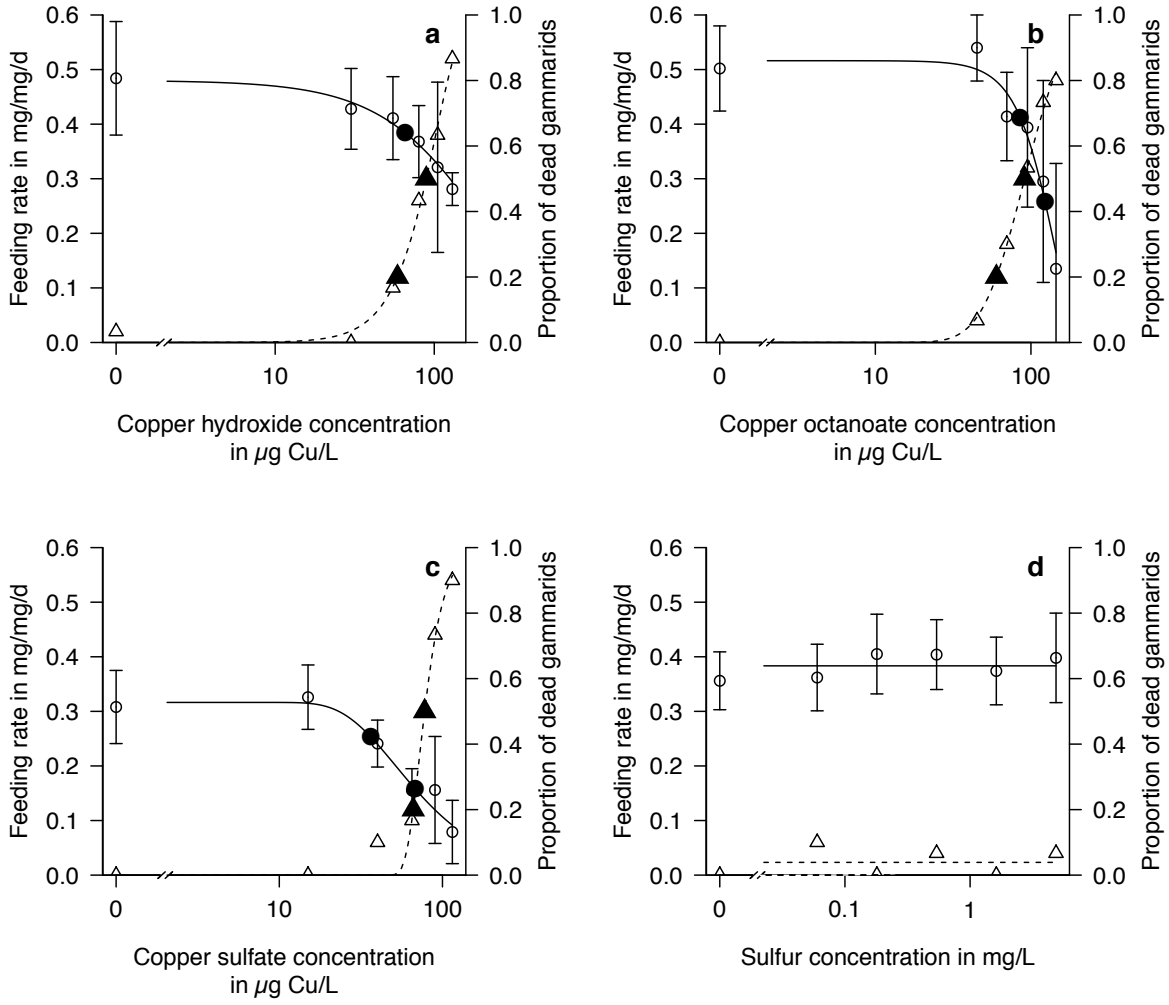

Fig. 3. Mean feeding rates (open circles) with 95% CIs of *G. fossarum* and proportion of dead animals (open triangles) when exposed to different concentrations of a) Cu-hydroxide, b) Cu-octanoate, c) Cu-sulfate, and d) sulfur. The best fitting models for feeding rate (solid line) and mortality (dashed line) as well as EC- (solid circles) and LC-values (solid triangles) for 20 and 50% are also displayed.

To the best of our knowledge, we observed for the first time an antagonistic effect of elemental sulfur on Cu-based pesticides' toxicity during the inorganic mixture experiment. This was evident from a lower effect of Cu-sulfate on survival (Fig. 4a) and feeding (Fig. 4b) than predicted by IA. For feeding not all differences were statistically significant due to high variability that was at least partly attributable to the reduced replication as a result of the considerable mortality at high concentrations.

However, these findings are supported by an alternative evaluation method. Considering also the variability associated with the IA prediction, this analysis uncovered statistically significant deviations of the modeled response (based on data from the mixture experiment) from the IA prediction – irrespective of the endpoint investigated – starting with a Cu-concentration of approximately 70 µg Cu/L (Fig. A3). The observed antagonism increased Cu-sulfate's LC_{50} by more than 20%, while feeding inhibition leveled-off at 38% effect (Table 3). As the addition of sulfur did not change the total Cu-concentration in the inorganic mixture experiment in comparison to the single substance Cu-sulfate bioassay (Table 2) – although an effect on Cu-speciation cannot be ruled out – it seems plausible that this effect is due to physiological processes within the test organisms. Accordingly, studies in nutritional science uncovered that sulfur can act as dietary antagonist of Cu in animals *inter alia* due to metabolization into sulfides that make Cu physiologically unavailable (e.g. Mills, 1985).

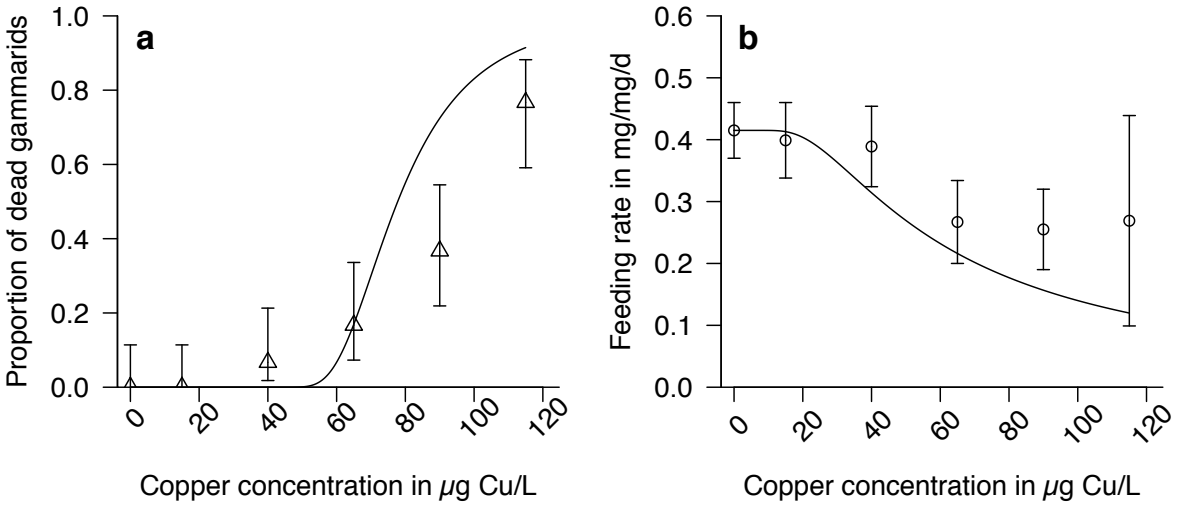

Fig. 4. Proportion of dead animals (triangles; a) and mean feeding rates (circles; b) of *G. fossarum* – with 95% CIs – when exposed to different concentrations of Cu-sulfate with a fixed concentration of sulfur (165 µg/L). The solid lines indicate predictions by IA.

RISK FOR *GAMMARUS*-MEDIATED LEAF LITTER BREAKDOWN ASSOCIATED WITH THE TESTED SUBSTANCES

The results of the present study emphasize the sensitivity of gammarids' feeding rate as ecotoxicological endpoint (*cf.* Maltby, 1994). The EC_{20}-values presented here are generally comparable to NOECs obtained by the chronic *D. magna* test (Table 1 and 3), the standard invertebrate assay for the environmental risk assessment of fungicides, which may, however, not always represent the endpoint used for risk

assessment during the process of substance registration. The only exceptions were carbendazim and tebuconazole, which are both known to directly affect reproduction (Taxvig et al., 2007; Miracle et al., 2011).

Besides being affected by chemical contaminants, feeding rates of gammarids depend on food-quality (e.g. Graça et al., 1993), which is particularly governed by the leaf-associated microbial community (e.g. Bundschuh et al., 2011). The composition of these microbial communities in turn varies with season (Nikolcheva and Bärlocher, 2005). As the present study was performed over the course of approximately one year, microbial inocula were prepared on several occasions and may thus have resulted in leaf discs of differing quality for the single experiments, which questions the repeatability and thus the reliability of our results. Accordingly, the control feeding rates differed substantially among the experiments (ranging from 0.2 to 0.5 mg/mg/d; Figs. 1-4). However, the sensitivity of *Gammarus* to the tested chemicals seemed to be much less affected: for each of four definitive experiments (i.e. carbendazim, Cu-sulfate, the organic fungicide mixture, and tebuconazole) one preliminary trial allowed the calculation of a second EC_{50}-value (data not shown). These preliminary experiments were conducted up to three months prior to the corresponding definitive tests resulting in a high average coefficient of variation (CV) of 25% (ranging from 11 to 38%) for the control feeding rates. At the same time, the EC_{50}-values varied with a four times lower average CV (6%; ranging from 3 to 12%) significantly less (paired *t*-test; $P = 0.027$, $n = 4$). This high level of repeatability indicates low implications of seasonal deviations in the microbial community associated with the inocula on gammarids' sensitivity. This statement is further underpinned by a study of Bundschuh and Schulz (2011) using the same experimental design as employed during the present study. There, two feeding assays were conducted three years apart from each other exposing gammarids to wastewater from the same treatment plant, which produced relative impairments in gammarids' feeding rate deviating by only 3% between the experimental runs. In fact, the repeatability of the *Gammarus* feeding assay thus seems to be comparable to highly standardized toxicity tests such as the acute *Daphnia* assay (e.g. CV of 5% for sodium chloride; as reviewed in Persoone et al., 2009). Therefore, the experimental design employed here, may be considered as a suitable ecotoxicological tool also for mixture studies and for use in risk assessments.

However, in order to assess the risk of the observed ecotoxicological effects in the field, EC_{20}-values may be used as endpoint. Applying this effect size seems

necessary given that *G. fossarum* males completely deplete their energy reserves during late summer (Becker et al., 2013), which regionally coincides with the highest measured fungicide concentrations (Battaglin et al., 2010). Thus, even a relatively low inhibition of feeding may cause deleterious effects on these animals. Furthermore, assessment factors are commonly used to extrapolate laboratory toxicity data to the field (European Food Safety Authority, 2013). A factor of at least ten applied to the EC_{20}-values seems necessary to account for instance for the fact that cryptic lineages (i.e. genetically distinct groups of organisms classified as one nominal species) of *G. fossarum* other than the one used in the present study can be up to six times more sensitive to chemical stressors such as tebuconazole (Feckler et al., 2012; 2013). By applying this factor, a risk at concentrations detected in surface waters (Table 1) was indicated for more than half of the tested fungicides. In addition, single substance EC_{20}- and EC_{50}-values were on average approximately three times higher than the fungicides' concentrations needed to obtain the organic mixture's EC_{20} and EC_{50} (Table 3). Accordingly, the organic mixture-EC_{20} occurred at a highly relevant total fungicide concentration (i.e. 61 µg/L), considering environmental concentrations of up to about 80 µg/L during run-off events (Bereswill et al., 2012; 2013). This situation is even more worrisome since although all tested organic fungicides have dissimilar intended modes of action, fungicides interacted synergistically in higher concentration ranges (see section 3.1; Fig. 2b). Therefore, it seems questionable if assessment factors – as applied above or during the environmental risk assessment of fungicides (European Food Safety Authority, 2013) – are sufficiently protective when considering complex mixtures (*cf*. Schäfer et al., 2012).

Moreover, also for the tested Cu-compounds, a risk of deleterious effects on gammarids in the field is apparent even without applying an assessment factor since Cu-concentrations from tens (entry via run-off; Bereswill et al., 2012; 2013) to hundreds of micrograms per liter (entry via spray drift; Süss et al., 2006) have already been detected in surface waters. Cu-concentrations of several hundreds of micrograms per liter may result in extirpation of amongst others *G. fossarum* for the Cu-compounds tested in the present study. But also concentrations in the tens of micrograms per liter range may result in lethal and sublethal effects on *Gammarus* (Table 3). Although the Cu-concentrations mentioned above are not necessarily measured in streams which are impacted by organic farming only, the present study suggests that also fungicide input from organic fields (besides that which arises from

conventional farming) may potentially affect the tested key shredder and consequently leaf litter breakdown. However, additional research is needed to assess the risk of Cu-exposure under field conditions in order to draw final conclusions. For instance the highly site-specific bioavailability of Cu for *Gammarus* (Bourgeault et al., 2013) needs to be considered, but also compensatory feeding behavior following peak exposures (Zubrod, personal observation) and its consequences for gammarids' physiological fitness. As shown in the inorganic mixture experiment, these assessments are further complicated by sulfur's potential to mitigate in part the effects of Cu on *Gammarus* (Figs. 4a and 4b), whereas binary mixtures of Cu and sulfur may still result in sublethal and lethal effects at field-relevant Cu-concentrations (Tables 1 and 3).

CONCLUSION

In this study we provide evidence that single fungicides and fungicide mixtures result in sublethal and lethal effects on the key shredder species *G. fossarum* at environmentally relevant concentrations. However, when considering repeated peak exposures (Bereswill et al., 2012; 2013) and the presence of multiple stressors (Holmstrup et al., 2010) in the field, effect concentrations may be even lower than observed here. In addition, leaf-associated fungal decomposers – on which shredders' nutrition heavily relies (Bärlocher, 1985) – can be affected by fungicide exposure (e.g. Bundschuh et al., 2011b), a fact acknowledged in the current guidance for aquatic environmental risk assessment (European Food Safety Authority, 2013). Leaf-shredding detritivores such as gammarids may consequently be subjected to combined effects (resulting from direct toxic and indirect, food-quality related effects; *cf*. Zubrod et al., 2011). Considering these pathways, fungicides may already impact decomposer-detritivore systems and consequently leaf litter breakdown in the field (*cf*. Schäfer et al., 2012). However, the situation may aggravate in the future as more intensive fungicide use may become necessary due to global climate change (Hakala et al., 2011) and the introduction of new fungal diseases such as soybean rust in the U.S. (Stokstad, 2004). Despite these warning signs, fungicides' impact on leaf-litter breakdown remains poorly studied and additional experimental effort – for instance regarding the relative importance of direct and indirect fungicide effects for shredders' physiological fitness – seems necessary.

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SUPPLEMENTARY MATERIAL

FIG. A1: CA PREDICTIONS FOR THE ORGANIC FUNGICIDE MIXTURE

The expected effect according to CA can be calculated as described by Backhaus et al. (2000):

$$ECx_{mix} = (\sum_{i=1}^{n} \frac{p_i}{ECx_i})^{-1} \qquad (1)$$

where ECx_{mix} is the total fungicide concentration of the mixture resulting in *x*% effect and p_i is the proportion of fungicide *i* in the mixture. However, due to the low mortality

in the bioassay with tebuconazole resulting in a maximum modeled proportion of dead animals of slightly higher than 7% within the tested concentration range, every prediction with a higher effect size would be specualtion. In addition, due to the baseline mortality (10%) observed in the bioassay with carbendazim, predicitions for effect sizes below the abovementioned 7% level are mathematically impossible. Thus no scientifically sound predictions according to CA were possible for mortality in the organic mixture experiment. Moreover, from equation 1 it is obvious that predictions by CA are mathematically restricted to the lowest maximum effect (i.e. feeding rate reduction) of the five organic fungicides. As cyprodinil's concentration-response curve (Fig. 1c) leveled-off at a rather low feeding rate reduction of 54%, predictions according to CA were also restricted to this maximum effect. In the predictable range (up to a total fungicide concentration of 96 µg/L), CA predictions agreed well with the observations (Fig. A1).

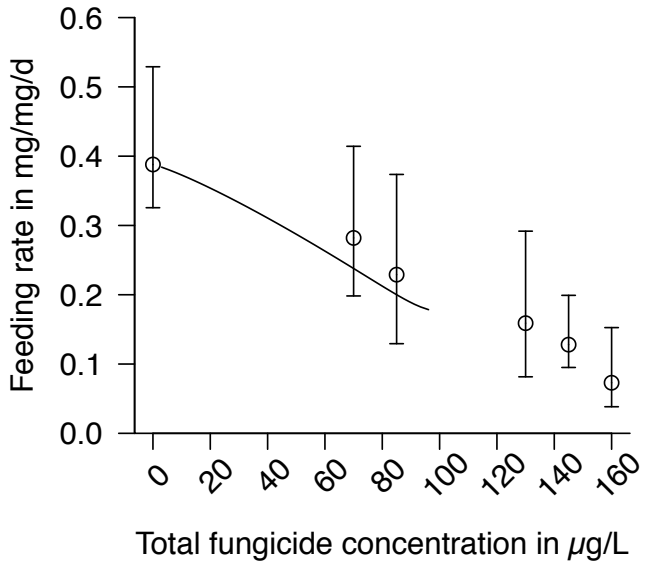

Fig. A1. Median feeding rates of *G. fossarum* – with 95% CIs – when exposed to different concentrations of the organic mixture. The solid line indicates predictions by CA.

FIG. A2: RANGE-FINDING EXPERIMENT FOR THE ORGANIC FUNGICIDE MIXTURE

The data presented originate from a range-finding experiment for the organic mixture and shows at 135 µg/L a statistically significantly higher effect on the feeding of *Gammarus* than predicted by IA (i.e. synergism; Fig. A2b). At concentrations, which were higher than tested in the final experiment, a significant synergistic effect on

mortality was also observed (Fig. A2a). Note that the reported concentrations are nominal and were not verified by chemical analysis.

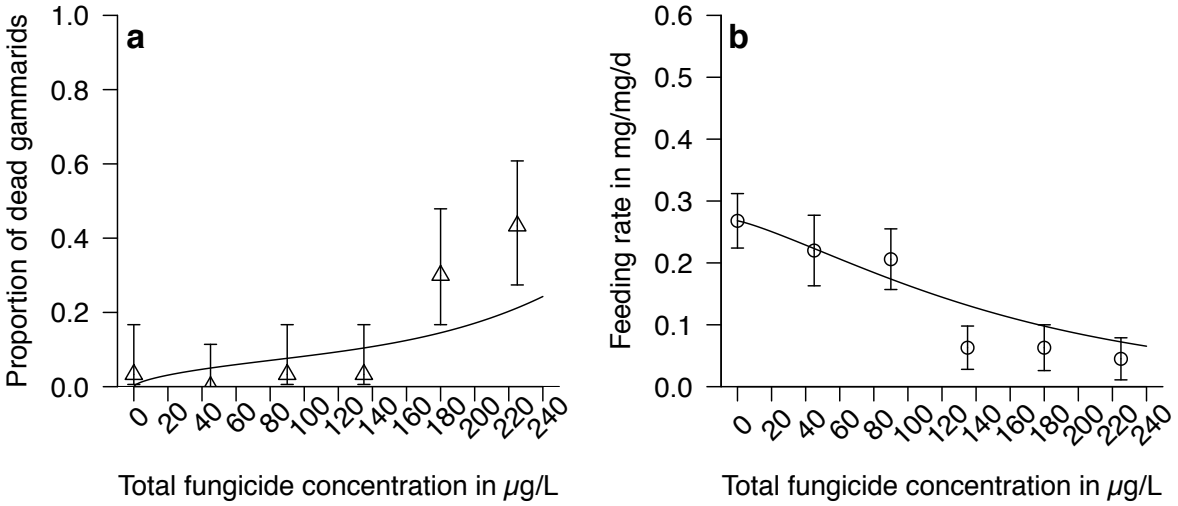

Fig. A2. Proportion of dead animals (triangles; a) and median feeding rates (circles; b) of *G. fossarum* – with 95% CIs – when exposed to different concentrations of the organic mixture. The solid lines indicate predictions by IA.

FIG. A3: ALTERNATIVE APPROACH TO ANALYZE THE COMPLIANCE OF THE INORGANIC MIXTURE'S TOXICITY WITH IA

In a binary mixture using one ineffective component (i.e. sulfur), the mixture effect according to IA (equation 2 in the main manuscript) reduces to the effect of the effective stressor (i.e. Cu-sulfate). In this present case, the concentration-response curve from the mixture experiment using Cu-sulfate and sulfur can be directly compared to the concentration-response curve from the bioassay which assessed Cu-sulfate singly while considering the variability associated with both curves. These were compared in µg Cu/L-steps using the "comped"-function available in the R extension package "drc" (Ritz and Streibig, 2005) based on the method described by Wheeler et al. (2006). By doing so, statistically significant differences ($P < 0.05$) between the curves from the single substance Cu-sulfate bioassay and the mixture experiment were revealed at Cu-concentrations ≥ 72 for mortality and in the concentration range from 35 to 39 µg Cu/L as well as at concentrations ≥ 73 µg Cu/L for feeding (Fig. A3).

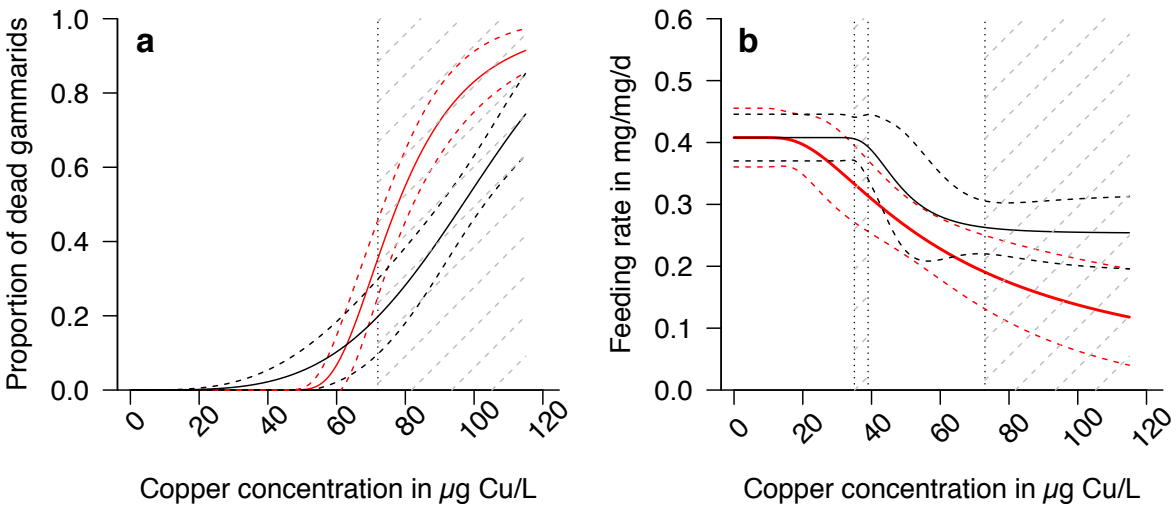

Fig. A3. Modeled proportion of dead animals (a) and feeding rate (b) of *G. fossarum* (solid black lines) – with 95% CIs (hatched black lines) – when exposed to different concentrations of Cu-sulfate with a fixed concentration of sulfur (165 µg/L). Red lines indicate predictions by IA with 95% CIs. Areas hatched in grey denote concentration ranges where curves differ statistically significantly.

TABLE A1. PHYSICO-CHEMICAL PARAMETERS OF SAM-5S

Table A1. Mean (with 95% CI) physico-chemical parameters of SAM-5S. Triplicate samples were measured from fresh, 3-d old (i.e. medium exchange during inorganic fungicide assays), and 7-d old (i.e. termination of organic fungicide assays) test medium.

	Fresh	3-d old	7-d old
pH	8.04 (8.00-8.08)	7.90 (7.76-8.05)	7.81 (7.76- 7.87)
Conductivity (µS cm^{-1})	397.5 (391.1-403.9)	416.0 (404.6-427.4)	429.0 (397.9-460.1)
Calcium (mg/L)	56.0 (51.0-61.0)	50.7 (43.1-58.3)	50.7 (44.9-56.4)
Magnesium (mg/L)	7.3 (0.0-15.3)	4.9 (1.8-7.9)	4.9 (0.0-10.1)
Chloride (mg/L)	76.7 (62.3-91.0)	5.5 (4.3-6.7)	5.7 (4.2-7.1)
Sulfate (mg/L)	<25	<25	<25
Sulfide (mg/L)	<0.05	<0.05	<0.05
Nitrate (mg/L)	<1	2.2 (0.3-4.1)	2.7 (1.2-4.1)
Nitrite (mg/L)	<0.005	0.023 (0.016-0.030)	0.037 (0.006-0.068)
Ammonium (mg/L)	<0.02	<0.02	<0.02
Ortho-Phosphate (mg/L)	<0.14	<0.14	<0.14

TABLE A2. PARAMETERIZATION OF DOSE-RESPONSE MODELS

Table A2. Models used for concentration-response-modeling and the respective parameterization[a].

Fungicide	Endpoint	Model	Parameters
Azoxystrobin	feeding	log-logistic (3 parameters with lower limit 0)	b=9.909465; d=0.207277; e=90.812731
	mortality	log-logistic (3 parameters with upper limit 1)	b=-3.743690; c=0.046749; e=152.374444
Carbendazim	feeding	log-logistic (3 parameters with lower limit 0)	b=-1.007142; d=0.313807; e=75.035943
	mortality	log-logistic (5 parameters)	b=-130.106976; c=0.114202; d=0.635465; e=54.964593; f=0.031256
Cyprodinil	feeding	Weibull (type 1 with 4 parameters)	b=1.438752; c=0.169186; d=0.374844; e=27.285081
	mortality	log-logistic (2 parameters)	b=-0.57132; e=836.09254
Quinoxyfen	feeding	log-logistic (3 parameters with lower limit 0)	b=1.006197; d=0.242463; e=23.834362
	mortality	Weibull (type 1 with 2 parameters)	b=-1.42740; e=86.34673
Tebuconazole	feeding	Weibull (type 1 with 3 parameters)	b=1.474526; d=0.301453; e=253.620495
	mortality	Weibull (type 1 with 2 parameters)	b=-0.16657; e=163960
Cu-hydroxide	feeding	log-logistic (4 parameters)	b=-1.269956; c=0.480038; d=-0.090288; e=230.821867
	mortality	Weibull (type 2 with 2 parameters)	b=2.72026; e=101.94476
Cu-octanoate	feeding	Weibull (type 1 with 3 parameters)	b=3.069990; d=0.516247; e=138.839929
	mortality	Log-normal (2 parameters)	b=2.01484; e=91.07518
Cu-sulfate	feeding	Weibull (type 2 with 3 parameters)	b=-1.348097; d=0.316378; 51.757924
	mortality	Weibull (type 1 with 2 parameters)	b=-5.23962; e=72.51468
Sulfur	feeding	linear regression without slope parameter	-
	mortality	linear regression without slope parameter	-
Organic mixture	feeding	Weibull (type 1 with 3 parameters)	b=1.670968; d=0.404880; e=148.762402
	mortality	Weibull (type 1 with 2 parameters)	b=-5.2866; e=182.7115
Inorganic mixture	feeding	Weibull (type 2 with 4 parameters)	b=-5.785257; c=0.253466; d=0.407957; e=45.150884
	mortality	Weibull (type 2 with 2 parameters)	b=3.88576; e=106.25642

[a] Parameterization according to Ritz and Streibig (2005)

TABLE A3. PARAMETERIZATION OF DOSE-RESPONSE MODELS

Table A3. NOEC-values for feeding rate and mortality derived from bioassays with single fungicides or mixtures using ANOVA followed by Dunnett's test or Kruskal-Wallis test followed by Bonferroni-adjusted Wilcoxon rank-sum-tests.

Fungicide	NOEC feeding rate	NOEC mortality
Azoxystrobin	90.0	90.0
Carbendazim	25.0	40.0
Cyprodinil	15.6	105.5
Quinoxyfen	9.0	≥52.5
Tebuconazole	92.5	≥585.7
Copper hydroxide	≥130.0	55.0
Copper octanoate	120.0	70.0
Copper sulfate	40.0	65.0
Sulfur	≥4,860.0	≥4,860.0
Organic mixture	85.0	≥160.0
Inorganic mixture	40.0	65.0

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APPENDIX A.2

Inorganic fungicides can increase microorganism-mediated palatability of leaves for a shredder but reduce microbial leaf decomposition

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SUMMARY

1. The application of fungicides is considered an indispensible measure to secure crop production. These substances, however, may unintentionally enter surface waters via runoff, potentially affecting the microbial community. To assess such risks adequately, authorities recently called for suitable test designs involving relevant aquatic microorganisms.

2. We assessed the structural and functional responses of leaf-associated microbial communities, which play a key role in the breakdown of allochthonous leaf material in streams, towards the inorganic fungicides copper (Cu) and elemental sulfur (S). These substances are of particular interest as they are authorized for both conventional and organic farming in many countries of the world. We used the food-choice of the amphipod shredder *Gammarus fossarum* (indicative for microorganism-mediated leaf palatability) as well as microbial leaf decomposition as functional endpoints. Moreover, the leaf-associated microbial communities were characterized by means of bacterial density, fungal biomass and community composition facilitating mechanistic understanding of the observed functional effects.

3. While *Gammarus* preferred Cu-exposed leaves over unexposed ones, microbial leaf decomposition was reduced by both Cu and S (up to 30%). Furthermore, Cu-exposure decreased bacterial densities (up to 60%), stimulated the growth of leaf-associated fungi (up to 100%) and altered fungal community composition, while S did not affect any of the assessed structural endpoints.

4. Synthesis and applications: We observed both structural and functional changes in leaf-associated microbial communities at inorganic fungicide concentrations realistic for surface water bodies influenced by conventional but also organic farming. Our data hence justify a careful reevaluation of the environmental safety of the agricultural use of these compounds. Moreover, inclusion of experimental designs similar to the one used in the present study in lower tier environmental risk assessment of antimicrobial compounds may aid to safeguard the integrity of aquatic microbial communities and the functions they provide.

KEYWORDS

antagonistic effect – antimicrobial – aquatic hyphomycetes – bacteria – biofilm – environmental risk assessment – ecosystem functioning – heavy metal – leaf litter breakdown – mixture toxicity

INTRODUCTION

Fungal pests are among the major threats to crop production. Although managed, due to these pathogens a portion of yield is lost annually, which is estimated high enough to feed more than 8% of the world's human population (Fisher et al., 2012). Thus, the use of chemical control agents (i.e. fungicides) is considered essential to secure global food supply (Strange and Scott, 2005). Driven by global change, the application of fungicides may even increase due to altered climatic conditions (Hakala et al., 2011) and invasive fungal pests (*cf*. Stokstad, 2004). However, these compounds contaminate freshwater ecosystems via drainage, spray drift, and surface run-off (Bereswill et al., 2012), where they may affect aquatic microorganisms and their functions. Despite these potential effects in the field, aquatic microorganisms are largely ignored during the environmental risk assessment of fungicides (Maltby et al., 2009). To close this gap, the European Food Safety Authority (EFSA; 2013) recently called for suitable test designs involving relevant aquatic microorganisms.

In this context, microorganisms involved in the decomposition of allochthonous organic matter (e.g. leaf litter) seem a suitable starting point since both leaf-associated bacteria and fungi are known to respond to a wide range of chemical stressors such as heavy metals (e.g. Duarte et al., 2008), micropollutants (e.g. antibiotics; Bundschuh et al., 2009) and pesticides (e.g. synthetic fungicides; Bundschuh et al., 2011). In addition, these organisms – particularly the polyphyletic group of fungi subsumed as aquatic hyphomycetes (Suberkropp and Klug, 1976) – accomplish two fundamental functions: firstly, they contribute considerably to leaf processing by direct decomposition (e.g. Hieber and Gessner, 2002). Secondly, they condition leaf material by increasing the leaves' nutrient content (e.g. proteins and lipids) and degrading recalcitrant structural leaf components; these transformations convert leaves into a more palatable and nutritious food source for leaf-shredding macroinvertebrates (Bärlocher, 1985). Any fungicide-induced effect on microorganisms present on decaying leaf material may thus have far-reaching consequences for the detritus-based food web due to its bottom-up regulation (Wallace and Eggert, 1997; Zubrod et al., 2011).

Among fungicides, inorganic substances such as copper (Cu)-based compounds and sulfur (S) represent a special case: firstly, they are routinely used in both conventional and organic farming (e.g. in Germany; BVL, 2013). Secondly, these substances show a general antimicrobial activity, i.e. they do not act specifically on

fungi (Flemming and Trevors, 1989; Williams and Cooper, 2004). Owing to the importance of Cu as a freshwater contaminant (e.g. Sridhar et al., 2005), some studies assessed leaf-associated microbial community composition and functioning in response to Cu-exposure (Duarte et al., 2008; Duarte et al., 2009b; Pradhan et al., 2011; Roussel et al., 2008; Tattersfield, 1993). However, results are partly contradictory and the tested Cu-concentrations were often far above levels relevant for agriculturally impacted streams (*cf*. Süss et al., 2006). In addition, little is known about the effects of elemental S as well as the joint effect of Cu and S on microorganisms involved in leaf litter breakdown.

Against this background, we tested a recently developed experimental design (Bundschuh et al., 2009; 2011) for its applicability during fungicide risk assessment, employing inorganic fungicides at realistic concentrations for agriculturally influenced surface waters. Three Cu-based fungicides (i.e. Cu-hydroxide, Cu-octanoate and Cu-sulfate) as well as elemental S were investigated in independent experiments. Moreover, since Cu and S are jointly applied (*sensu* Jamar et al., 2010), a binary mixture composed of the Cu-compound causing the strongest effect on fungal biomass and S was tested. Leaf material that was microbially colonized under control conditions or in the presence of fungicides was used to assess two functional endpoints: firstly, the food-choice of *Gammarus fossarum* KOCH (Crustacea, Amphipoda), a key shredder in low order streams (Englert et al., 2013), was utilized to demonstrate fungicide-induced alterations in microorganism-mediated leaf palatability. Secondly, microbial leaf decomposition was quantified. Finally, leaf-associated microbial communities were characterized to facilitate mechanistic understanding of potential functional effects. We hypothesized that (i) due to their general antimicrobial activity, exposure to Cu and S would result in effects on both leaf-associated bacteria and fungi, while we expected (ii) all tested Cu-compounds to cause comparable effect patterns as a result of their common major toxic cations (i.e. Cu^{2+} and $CuOH^{+}$; Flemming and Trevors, 1989). Moreover, we (iii) anticipated both assessed functional endpoints to be negatively affected by fungicide exposure (*cf*. Tattersfield, 1993) and (iv) an antagonistic mixture effect of Cu and S in leaf-associated microbial communities (*cf*. Zubrod et al., 2014).

MATERIALS AND METHODS

SOURCES OF LEAF MATERIAL, FUNGAL INOCULUM AND GAMMARIDS

Black alder (*Alnus glutinosa* (L.) GAERTN.) leaves were collected in October 2011 near Landau, Germany (49°11'N; 8°05'E) and stored at -20°C until further processing. Following in general the procedure described by Bundschuh *et al.* (2011), for each of the five experiments, 500 leaves were deployed for 14 days in fine-mesh bags (0.5 mm mesh size; 10 leaves per bag) in the Rodenbach near Grünstadt, Germany (49°33'N, 8°02'E; step 1 in Fig. 1), upstream of any agricultural activity, settlement, and wastewater inlet. In the laboratory, another 500 leaves were added to the retrieved leaf material (for retrieval dates see Table 1) and thoroughly mixed (step 2 in Fig. 1). Leaves were kept in aerated nutrient medium (Dang et al., 2005; referred to as "conditioning medium"), containing high amounts of nitrate and phosphate to prevent nutrient limitation of microorganisms, at 16±1°C in total darkness for another 14 days (medium exchange after 7 days). Hence, inoculum with leaves featuring microbial communities at varying successional stages and thus a presumed high diversity of aquatic hyphomycetes was produced (*cf*. Gessner et al., 1993).

Table 1. Applied products, supplier, nominal concentrations and dates on which inocula used in the five experiments were retrieved from the field.

Experiment	Product(s) applied	Supplier	Nominal concentrations	Retrieval of inoculum
Cu-hydroxide	Cuprozin flüssig	Spiess-Urania Chemicals	10, 50, 250, 500 µg Cu L^{-1}	Nov-2011
Cu-octanoate	Cueva Pilzfrei	W. Neudorff GmbH KG	10, 50, 250, 500 µg Cu L^{-1}	Jan-2012
Cu-sulfate	Pentahydrate salt	Fluka	10, 50, 250, 500 µg Cu L^{-1}	Dec-2011
S	Kumulus WG	BASF SE	0.05, 0.2, 0.8, 3.2 mg S L^{-1}	May-2012
Binary mixture	See above	See above	10, 50, 250, 500 µg Cu L^{-1} + 0.2 mg S L^{-1}	Jun-2012

Seven days prior to their use in food-choice experiments, *G. fossarum* were kick-sampled in the Hainbach, Germany (49°14'N; 8°03'E; upstream of any agricultural activity, settlement, and wastewater inlet), whose population is exclusively composed of cryptic lineage B (Feckler et al., 2014). Only adult males of approximately 6 to 8 mm body length and visually free of acanthocephalan parasites were used. Throughout the acclimation phase in the laboratory, animals were kept in a temperature-controlled chamber at 20±1°C in total darkness, while they were gradually adapted to SAM-5S amphipod medium (Borgmann, 1996). Gammarids

were fed *ad libitum* with pre-conditioned black alder leaves for the first 3 days, but were starved for the remaining 4 days to level their appetite.

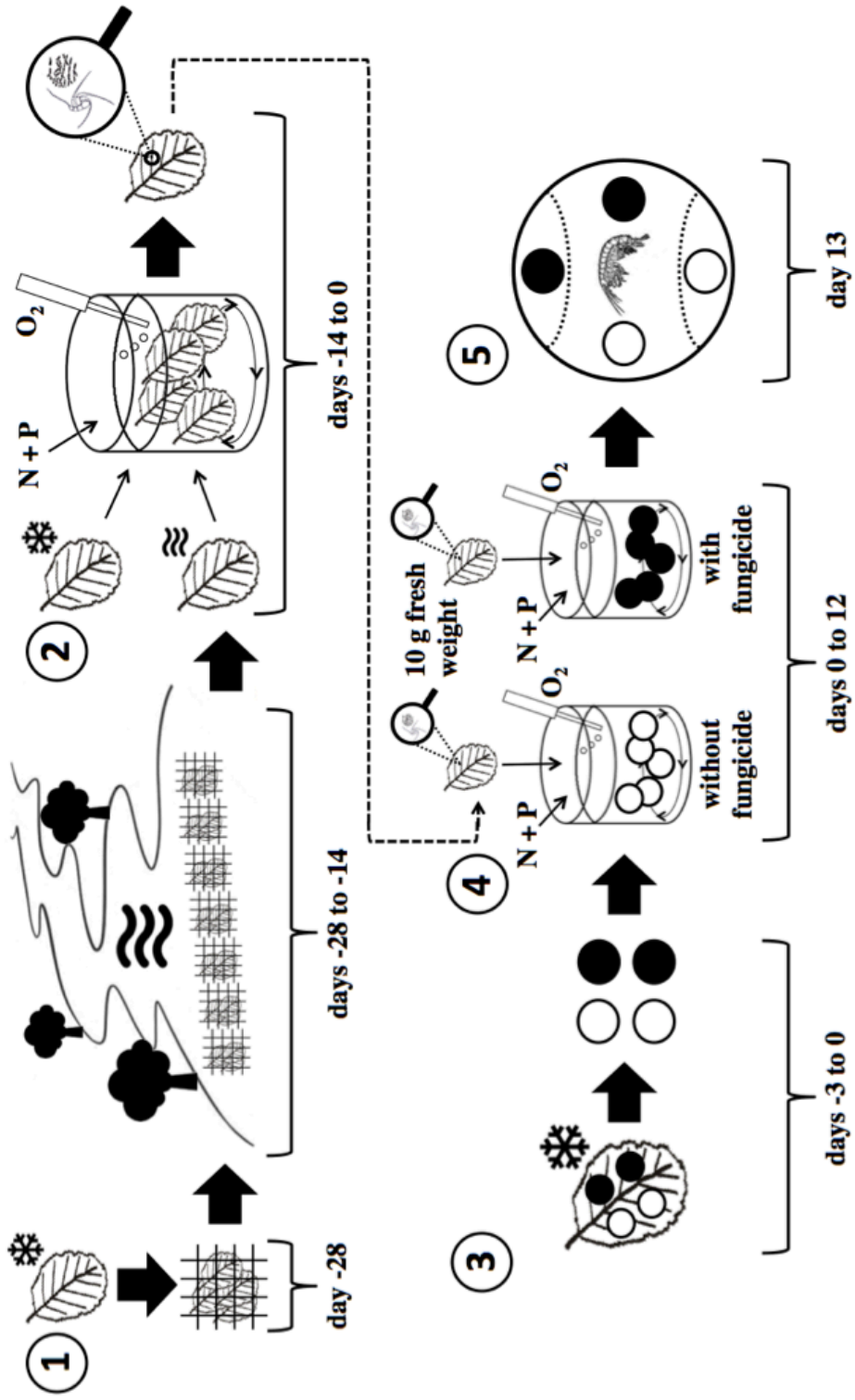

Fig. 1. Scheme illustrating the experimental design of the present study. Step 1: Field-exposure of 500 leaves. Step 2: Field-exposed and 500 unexposed leaves are mixed and kept in nutrient medium to produce microbial inoculum. Step 3: Sets of four discs are cut out of unconditioned leaves. Step 4: Two discs per set are conditioned in the presence and two in the absence of fungicide(s) using 10-g of microbial inoculum per aquarium. Step 5: Food-choice experiment with *G. fossarum*. See text for more details.

TESTED FUNGICIDE CONCENTRATIONS

Conditioning of leaf material (see below) took place either under control conditions or in the presence of one of four fungicide concentrations. Nominal Cu-concentrations (Table 1) were selected to cover the range of concentrations reported for agriculturally influenced surface waters (up to 263 µg L^{-1}; Süss et al., 2006). To the best of the authors' knowledge, no reports about the contamination of surface waters with elemental S due to agricultural activities exist. Thus, a maximum predicted environmental concentration (PEC; 165 µg L^{-1}) was derived from FOCUS exposure modelling (FOCUS, 2001), ignoring the maximum solubility of elemental S in water (i.e. 63 µg L^{-1}; FOOTPRINT, 2014). This was done because naturally occurring solvents can increase water solubility of plant protection products (Chiou et al., 1986). Nominal S-concentrations were selected to cover a range of approximately one third to twenty-fold the maximum PEC (Table 1). Moreover, to test the joint effect of Cu-hydroxide (the fungicide with the strongest effect on fungal biomass; see Results) and S, a mixture experiment was conducted using an "A in the presence of B"-design (Jonker et al., 2011). All Cu-hydroxide concentrations tested in the single substance experiments (Table 1) were applied in combination with a fixed concentration of 0.2 mg S L^{-1}, which approximates the maximum PEC.

LEAF CONDITIONING

In general, the experimental procedures followed Bundschuh *et al.* (2011) with slight alterations: To assess the two functional endpoints (leaf palatability and microbial leaf decomposition), sets of four leaf discs (diameter = 16 mm) were cut out of single thawed, unconditioned leaves (step 3 in Fig. 1). Discs were dried at 60°C for 24 h and weighed individually to the nearest 0.01 mg. Two discs per set were subjected to the control treatment and the remaining two to one of the four fungicide concentrations. These leaf discs and additional discs for the characterization of the leaf-associated microbial communities (see below) were placed together in 5-L glass aquaria (seven per concentration). Aquaria were located in a temperature-controlled chamber at 16±1°C (total darkness) and filled with 4 L conditioning medium containing the respective fungicide concentration. Ten grams fresh weight (accuracy: 0.1 g) of the microbially colonized leaves (described above) were added to each aquarium as microbial inoculum. Aquaria were continuously aerated and placed on magnetic stirrers simulating flow and securing a permanent homogenous distribution of the fungicides, fungal spores and bacterial cells (step 4 in Fig. 1). During the

conditioning phase, test solutions were renewed every 3 days, ensuring a continuous fungicide exposure (*cf*. Zubrod et al., 2014).

LEAF PALATABILITY AND MICROBIAL LEAF DECOMPOSITION

After 12 days, the leaf discs for the assessment of the two functional endpoints were rinsed for 30 min in fungicide-free SAM-5S to remove potentially adsorbed fungicide residues. One of the discs conditioned in the control and one conditioned in the presence of a fungicide, originating from the same leaf, were fixed in the centre of a food-choice arena (Bundschuh et al., 2009), i.e. a 300-mL crystallization dish filled with 100 mL fungicide-free SAM-5S (step 5 in Fig. 1). One gammarid was allowed to feed on these discs for 24 h (20±1°C in total darkness). During that time, the two corresponding discs from the same leaf were placed in the same arena but protected from amphipod feeding by 0.5-mm fiberglass mesh screen; these discs were used to control for abiotic as well as microbial leaf mass losses when calculating the leaf consumption of *Gammarus* and to determine microbial leaf decomposition. After 24 h, the amphipods as well as the remaining leaf material were dried and weighed as described above. Our initial experimental design with 49 replicates per fungicide concentration was on average reduced to 45 (ranging from 40 to 49) after removing those replicates where gammarids had died or molted.

CHARACTERIZATION OF THE MICROBIAL COMMUNITIES ON LEAVES

Microbial communities were characterized on leaf discs (one sample per conditioning aquarium; n = 7) conditioned together with the discs used in the food-choice experiments. Bacterial cell numbers associated with the leaf discs were determined according to Buesing (2005). Briefly, three leaf discs per aquarium were combined and preserved in formalin. After detaching bacteria from the leaf discs using ultrasonication (Sonopuls HD 2070 equipped with a TT 13 tip, both Bandelin, Berlin, Germany), a 5-µL aliquot was filtered on an aluminium oxide filter (pore size = 0.2 µm, Anodisc, Whatman, Beckenham, UK) and cells were stained with SYBRGreen II (Molecular Probes, Eugene, OR, USA). Digital photographs were taken with an epifluorescence microscope and the mean number of bacteria on twenty microscopic fields (accounting for ≥400 cells) was determined (Axio Scope.A1, AxioCam MRm and Axio-Vision Rel. 4.8, Carl Zeiss MicroImaging, Jena, Germany). Bacterial counts were divided by the dry weight of the leaf discs.

To quantify the ergosterol content of the leaf material (a proxy for fungal biomass) according to Gessner (2005), 15 leaf discs per aquarium were combined to a sample. Ergosterol was extracted from freeze-dried and ground discs in alkaline methanol and, following purification by solid-phase extraction (Sep-Pak® Vac RC tC18 500 mg sorbent, Waters, Milford, MA, USA), quantified by high-performance liquid chromatography (1200 Series, Agilent Technologies, Santa Clara, CA, USA) using a LiChrospher® 100 RP 18-5 m column (250.0 mm x 4.6 mm, particle size 5 mm, CS-Chromatographie Service, Langerwehe, Germany).

The aquatic hyphomycete community on the leaves was characterized on three leaf discs per aquarium in general according to Bärlocher (1982). Leaf discs were placed in wells of six-well plates (Cellstar® 6 Well Cell Culture Multiwell Plates, Greiner Bio-One, Frickenhausen, Germany) filled with 4 mL of deionized water and incubated on an orbital shaker set at 75 rpm. We did not add Cu and S during incubation, which may have resulted in some recovery of the leaf-associated microbial community. This more conservative approach was chosen to determine all aquatic hyphomycete species able to reproduce and disperse after fungicide-exposure – an ecologically highly relevant information (*cf*. Duarte et al., 2008). Moreover, incubation of leaf discs in the wells took place at a 4°C higher temperature compared to the discs' conditioning (i.e. at 20°C). Although aquatic hyphomycetes can differ in their temperature optima (e.g. Duarte et al., 2013), Bärlocher et al. (2013) found that the temperature at which leaves were incubated for spore assessment does not affect community composition significantly, suggesting no meaningful implications in the outcomes of the present study. After 96 h of incubation, fungal spores were fixed and stained by adding 0.5 mL lactophenol cotton blue. Spores were identified and counted at 100-fold magnification using several identification keys (mainly Ingold, 1975). Total number of spores was divided by the dry weight of the leaf discs.

CHEMICAL ANALYSES

Directly following the fungicide application and at the time of the first medium renewal, triplicate 10 mL-samples of the treatments with the lowest and the highest tested fungicide concentrations as well as one sample from the control treatments were collected. Cu-concentrations were measured using a quadrupole inductively coupled plasma-mass spectrometry (XSeries II, Thermo Fisher Scientific, Waltham, MA, USA) according to Rosenfeldt *et al.* (2014). Fungicide-free medium had a mean Cu-concentration of 0.75 (0.34-1.17; 95% confidence interval (CI); n = 4) and 0.51

(0.46-0.56) µg Cu L^{-1} shortly after application and at the medium renewal, respectively. For Cu-octanoate and Cu-sulfate, nominal and measured initial concentrations agreed well, but for Cu-hydroxide, measured initial concentrations were only about 60% of the nominal ones (see Appendix S1, Supporting Information).

The conditioning medium contained substantial levels of covalently bonded S (due to 3-(N-morpholino)propanesulfonic acid; *cf*. Dang et al., 2005). Since these would mask elemental S, S-concentrations arising from fungicide application (<5% of total S in the highest tested concentration) were not verified by chemical analysis. Physicochemical characteristics of fresh and 3-days old conditioning medium are provided in the Supporting Information (Appendix S1).

STATISTICAL ANALYSES

Leaf consumption during the food-choice experiments in mg leaf dry wt mg^{-1} gammarid dry wt day^{-1} was calculated for each accessible leaf disc according to Bundschuh *et al.* (2009). Microbial leaf decomposition (*D*) in mg leaf dry wt day^{-1} was determined using the leaf discs, which were protected from amphipod feeding, and was calculated as $D = (n_b * k - n_a)/t$. There, n_b and n_a are the initial and final dry weight of the leaf discs, respectively, *k* is an empirically derived, dimensionless factor (=0.74) controlling for leaching of the leaf material, which was determined by retrieving 15 pre-weighed leaf discs after 24 h from conditioning aquaria, and *t* is decomposition time (i.e. 13 days).

Univariate data were checked for normality by visual inspection, while homoscedasticity was tested via Levene's test. Paired data (food-choice and microbial leaf decomposition) were assessed for statistical significance using paired *t*-tests or – if the assumptions for parametric testing were not met – Wilcoxon signed-rank tests. Unpaired data (bacterial density, fungal biomass, number of fungal species and spore production) were analysed by performing ANOVAs followed by Dunnett's tests. As a non-parametric alternative, Kruskal-Wallis tests were applied followed by Wilcoxon rank-sum tests using the Bonferroni adjustment for multiple comparisons (for all univariate analysis see Zar, 2010).

Shifts in aquatic hyphomycete community composition were assessed with permutational multivariate analysis of variance (PERMANOVA) using square-root transformed data (to reduce the effect of dominant species; Clarke and Warwick, 2001) and Bray-Curtis similarities. Species found in only one sample were removed

from the analyses. Results of additional analyses and visualisations (non-metric multidimensional scaling ordination plots) are provided in the Supporting Information (Appendix S2). R version 3.0.2 for Mac (R Development Core Team, 2015) together with the add-on packages ”multcomp”, “plotrix” and ”vegan” was used for statistics and figures.

RESULTS

LEAF PALATABILITY

Leaf material exposed to 500 µg Cu L^{-1} for Cu-hydroxide (paired *t*-test; $P = 0.027$; Fig. 2a) and Cu-sulfate (paired *t*-test; $P = 0.040$; Fig. 2c) resulted in higher consumption by *G. fossarum*. Cu-octanoate caused a significant preference at 250 µg Cu L^{-1} (paired *t*-test; $P = 0.046$; Fig. 2b), whereas Cu-hydroxide resulted in a nearly significant preference (paired *t*-test; $P = 0.081$; Fig. 2a). S did not alter leaf preference by *G. fossarum* over all concentrations tested (Fig. 2d). Since 0.2 mg S L^{-1}, i.e. the S-concentration applied in the binary mixture (Table 1), had no effect on the remaining endpoints (see below), we analysed statistically if its addition in the course of the binary mixture experiment nevertheless affected the biological effects (in terms of the effect size) of Cu-hydroxide. Therefore, the data from the fungicide treatments of the binary mixture assay and the single substance experiment with Cu-hydroxide were normalized by the respective control mean and tested for significant differences using unpaired *t*-tests or Wilcoxon rank-sum tests. This approach is analogue to testing for compliance with the reference model “independent action”, which assumes mixture components to have dissimilar modes of action (Bliss, 1939), for the case of one component being ineffective, while including the variability associated with both prediction and observation (*cf*. Zubrod et al., 2014). This analysis revealed that the observed effect sizes were significantly lower at 250 (Wilcoxon rank-sum test, $P < 0.001$; Fig. 2a,e) and 500 µg Cu L^{-1} (Wilcoxon rank-sum test, $P = 0.004$; Fig. 2a,e) when Cu-hydroxide was in mixture with S. Nevertheless, the binary mixture resulted in a significant preference for fungicide exposed leaf material at 500 µg Cu L^{-1} (paired *t*-test; $P = 0.029$; Fig. 2e).

MICROBIAL LEAF DECOMPOSITION

Microbial decomposition was reduced by up to 30% in the presence of inorganic fungicides. Significant reductions were found at 250 and 500 µg Cu L^{-1} for Cu-hydroxide (Wilcoxon signed-rank tests, $P < 0.001$; Fig. 3a) and Cu-octanoate (paired

t-tests, $P < 0.001$; Fig. 3b), while all tested Cu-sulfate concentrations reduced decomposition significantly (paired *t*-tests, $P < 0.01$; Fig. 3c). Also at S-concentrations of 0.8 and 3.2 mg L^{-1} significant reductions in microbial leaf decomposition were observed (Wilcoxon signed-rank tests, $P < 0.01$; Fig. 3d). In contrast, when S was added, Cu-hydroxide resulted in a significantly increased

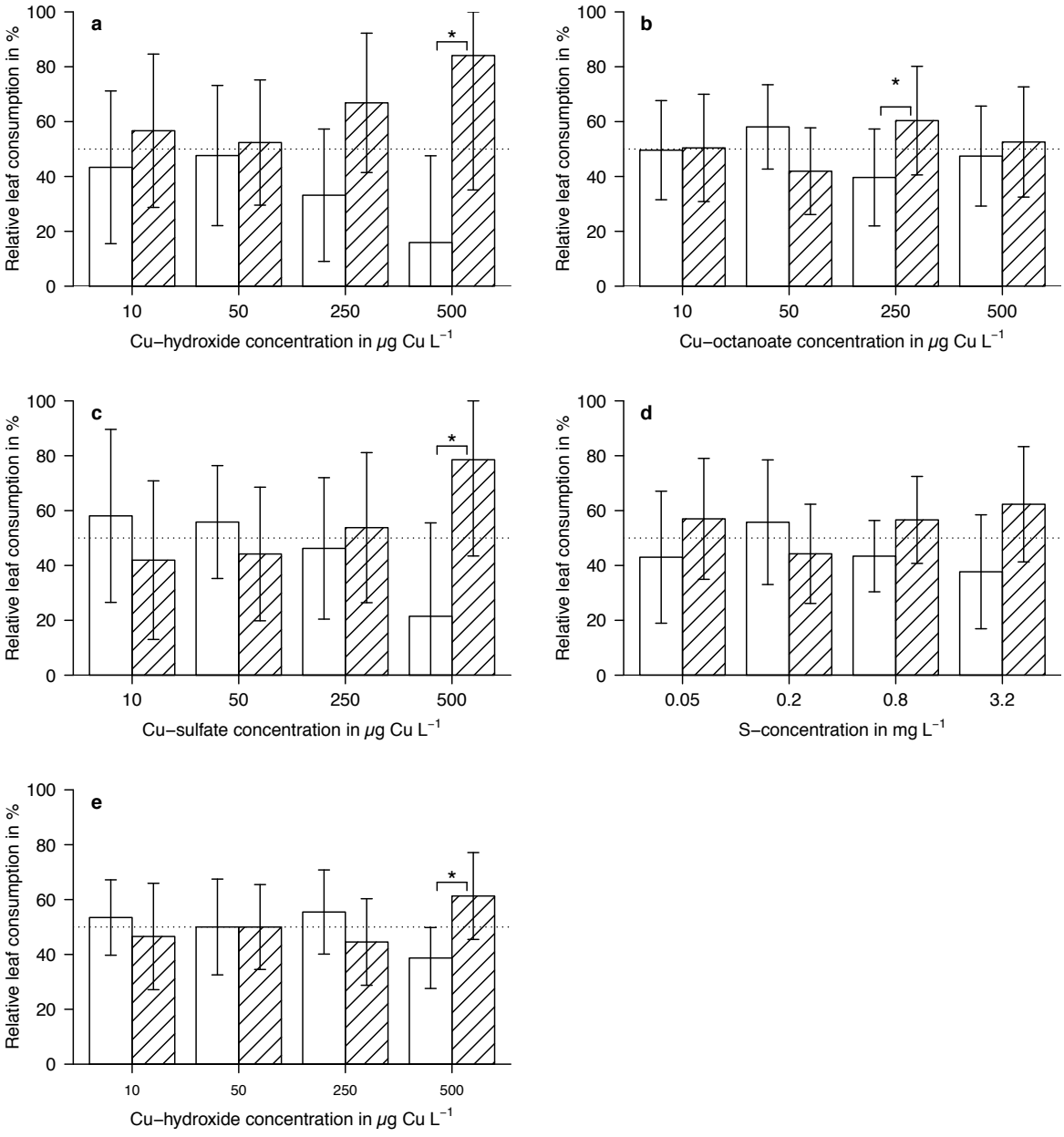

Fig. 2. Mean relative consumption (with 95% CI) of *G. fossarum* on leaf material conditioned either under control conditions (white bars) or in the presence of different concentrations of a) Cu-hydroxide, b) Cu-octanoate, c) Cu-sulfate, d) S and e) Cu-hydroxide amended by 0.2 mg S L^{-1} (hatched bars). Asterisks denote statistically significant differences. While 100% is the total leaf consumption in a food-choice experiment, the horizontal dotted line indicates the no-effect level (50% consumption of both leaf types). Group means or medians on the original scale are provided in Appendix S3.

microbial decomposition at 250 µg Cu L^{-1} (paired *t*-test, $P = 0.014$; Fig. 3e). Accordingly, reductions in microbial decomposition caused by Cu-hydroxide were significantly lower at 250 (Wilcoxon rank-sum test, $P < 0.001$; Fig. 3a,e) and 500 µg Cu L^{-1} (unpaired *t*-test, $P < 0.001$; Fig. 3a,e) when in mixture with S compared to the single substance experiment.

Table 2. Mean or median (depending on whether or not data of an experiment were normally distributed) number of species and number of spores. Reported *P*-values stem from Dunnett's tests or Bonferroni-adjusted Wilcoxon-rank sum tests for comparisons with the respective control. All *P*-values < 0.05 are printed in bold.

Experiment	Concentration	Number of species per sample	*P-value*	Number of spores mg^{-1} leaf	*P*-value
	0 µg Cu L^{-1}	5.7 (4.8-6.6)		4.9 (1.6-31.4)	
	10 µg Cu L^{-1}	5.7 (4.7-6.7)	1.000	9.1 (2.2-17.2)	1.000
Cu-hydroxide	50 µg Cu L^{-1}	5.1 (4.2-6.1)	0.842	8.3 (2.2-24.1)	1.000
	250 µg Cu L^{-1}	5.6 (4.2-7.0)	0.999	5.9 (1.7-10.8)	1.000
	500 µg Cu L^{-1}	5.9 (4.1-7.6)	0.999	10.3 (2.4-21.0)	1.000
	0 µg Cu L^{-1}	7.3 (6.1-8.4)		7.0 (4.3-41.0)	
	10 µg Cu L^{-1}	7.4 (5.8-9.1)	1.000	9.6 (1.7-64.2)	1.000
Cu-octanoate	50 µg Cu L^{-1}	7.0 (5.5-8.5)	0.993	6.0 (2.1-8.4)	1.000
	250 µg Cu L^{-1}	9.1 (7.2-11.1)	0.152	6.4 (1.6-22.7)	1.000
	500 µg Cu L^{-1}	9.1 (7.7-10.6)	0.152	23.3 (2.1-66.0)	1.000
	0 µg Cu L^{-1}	6.6 (5.7-7.5)		4.8 (3.8-14.1)	
	10 µg Cu L^{-1}	7.0 (6.2-7.8)	0.927	5.1 (3.3-17.6)	1.000
Cu-sulfate	50 µg Cu L^{-1}	6.9 (5.5-8.2)	0.982	7.3 (1.6-19.6)	1.000
	250 µg Cu L^{-1}	8.1 (6.5-9.8)	0.099	17.9 (1.4-32.4)	0.638
	500 µg Cu L^{-1}	9.7 (8.6-10.9)	**<0.001**	15.1 (5.5-37.6)	0.060
	0 mg L^{-1}	11.3 (9.9-12.7)		225.6 (28.8-495.6)	
	0.05 mg L^{-1}	10.0 (9.1-10.9)	0.340	178.3 (61.6-375.9)	1.000
S	0.2 mg L^{-1}	10.4 (9.1-11.7)	0.677	169.7 (106.5-339.5)	1.000
	0.8 mg L^{-1}	12.3 (10.9-13.7)	0.556	129.3 (14.2-178.1)	1.000
	3.2 mg L^{-1}	11.9 (10.0-13.7)	0.890	130.9 (38.9-384.2)	1.000
	0 µg Cu L^{-1}	10.9 (9.2-12.5)		161.3 (120.9-317.6)	
	10 µg Cu L^{-1}	8.3 (7.6-9.0)	**0.018**	144.6 (73.2-232.3	1.000
Binary mixture	50 µg Cu L^{-1}	9.1 (8.0-10.4)	0.160	212.4 (43.3-567.3)	1.000
	250 µg Cu L^{-1}	11.3 (9.1-13.5)	0.963	301.0 (135.2-538.6)	0.513
	500 µg Cu L^{-1}	10.7 (9.6-11.9)	0.999	538.8 (269.0-1411.3)	**0.016**

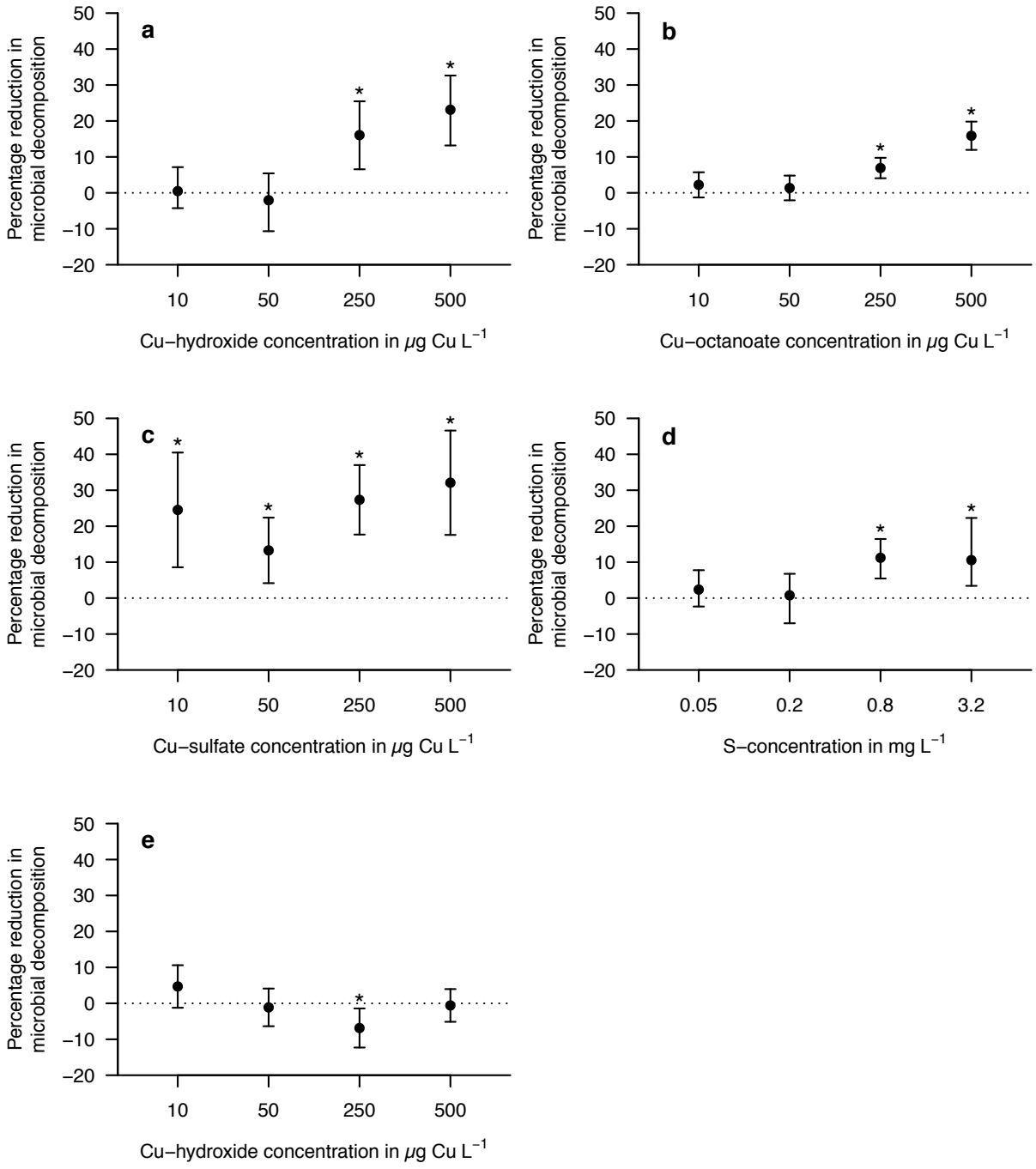

Fig. 3. Mean or median (depending on whether or not data of an experiment were normally distributed; with 95% CI) percentage reduction (compared to the respective control) in microbial decomposition of leaf material conditioned in the presence of different concentrations of a) Cu-hydroxide, b) Cu-octanoate, c) Cu-sulfate, d) S and e) Cu-hydroxide amended by 0.2 mg S L^{-1}. Asterisks denote statistically significant differences to the respective control. Group means or medians on the original scale are provided in Appendix S3.

MICROBIAL COMMUNITIES ON LEAVES

Cu-based fungicides reduced the bacterial density on exposed leaf material relative to the control by up to 60%. However, only for Cu-octanoate (at all Cu-

concentrations; Dunnett's test; $P < 0.05$; Fig. 4b) and Cu-sulfate (at 10 µg Cu L^{-1}; Dunnett's test; $P = 0.032$; Fig. 4c) were significant reductions found. Nevertheless, a strong tendency to a reduced bacterial density was also observed at 500 µg Cu L^{-1} in the experiment with Cu-hydroxide (30% reduction; Dunnett's test; $P = 0.084$; Fig. 4b). S did not have any statistically significant effect on bacterial densities at the concentrations tested (Fig. 4d) but significantly reduced Cu-hydroxide's effect on bacterial density at 250 µg Cu L^{-1} (by 26%; unpaired *t*-test; $P = 0.015$; Fig. 4a,e) compared to the single substance experiment. Also at 500 µg Cu L^{-1} a strong tendency to a reduced effect size was evident (by 14%; unpaired *t*-test; $P = 0.086$; Fig. 4a,e).

In the case of Cu-hydroxide and Cu-octanoate, fungal biomass increased with increasing Cu-concentration up to 100% with all (Bonferroni-adjusted Wilcoxon rank-sum tests; $P < 0.05$; Fig. 4a) or the two highest Cu-concentrations (Dunnett's test; $P < 0.01$; Fig. 4b), respectively, being significantly different from the control. No such increase was observed for Cu-sulfate (Fig. 4c), whereas during this experiment a 60% higher control mean fungal biomass relative to the average of the remaining controls was measured (Table S5). This exceptionally high control fungal biomass was probably due to the contamination with an unidentified fungus, which was evidenced by orange spots on the leaf discs of this experiment (Zubrod, personal observation), and may have masked effects on the remaining fungal community. Furthermore, while S (Fig. 4d) did not result in any significant differences compared to the control, the binary mixture of Cu-hydroxide and S caused significantly increased fungal biomasses at the two highest Cu-concentrations (Dunnett's test; $P < 0.01$; Fig. 4e). However, at 10 (unpaired *t*-test, $P = 0.005$; Fig. 4a,e) and 50 µg Cu L^{-1} (Wilcoxon rank-sum test, $P = 0.026$; Fig. 4a,e) the increase was significantly lower compared to the single substance experiment with Cu-hydroxide.

With the exception of Cu-octanoate, the tested Cu-based fungicides – singly and in combination with S – caused the community composition of leaf-associated aquatic hyphomycetes at 500 µg Cu L^{-1} to differ significantly from the respective control (PERMANOVA; $P < 0.05$; Fig. S1). But also for Cu-octanoate a near-significant difference was observed at 500 µg Cu L^{-1} (PERMANOVA; $P = 0.064$), while community composition differed significantly from the control at 50 µg Cu L^{-1} (PERMANOVA; $P = 0.016$). S caused no significant effect. Cu-sulfate (at 500 µg Cu L^{-1}) resulted in a significantly higher number of fungal species compared to the

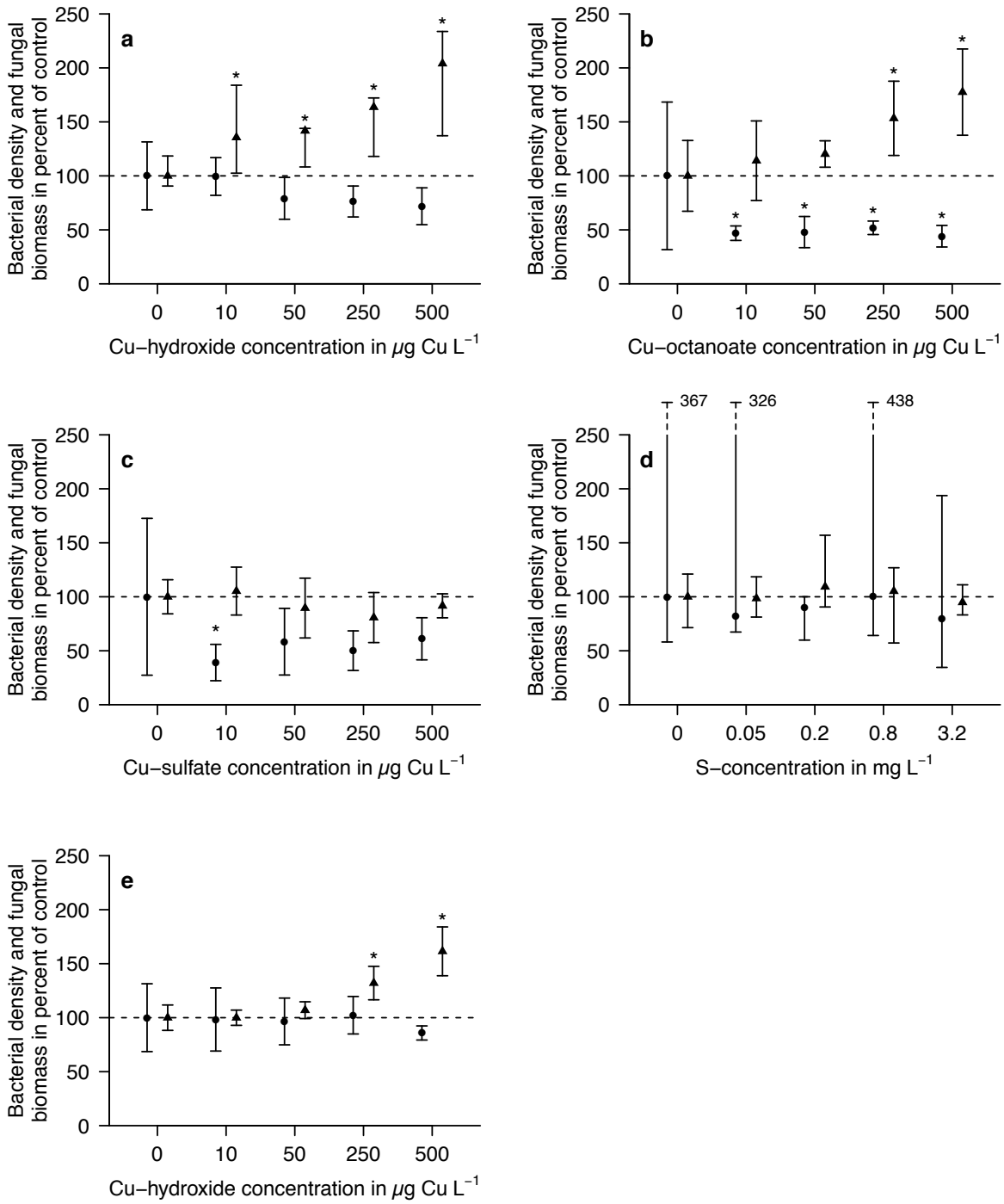

Fig. 4. Mean or median (depending on whether or not data of an experiment were normally distributed; with 95% CI) bacterial density (circles) and fungal biomass (triangles) relative to the respective control associated with leaf material conditioned in the presence of different concentrations of a) Cu-hydroxide, b) Cu-octanoate, c) Cu-sulfate, d) S and e) Cu-hydroxide amended by 0.2 mg S L^{-1}. Asterisks denote statistically significant differences to the respective control. Group means or medians on the original scale are provided in Appendix S3.

control (Table 2). While neither for Cu-hydroxide nor for S a significant effect was observed, the binary mixture of these substances resulted in a significant reduction at

10 µg Cu L^{-1} (Table 2). At this concentration of the mixture, the number of species relative to the control was also significantly lower compared to the single substance experiment with Cu-hydroxide (unpaired *t*-test; *P* = 0.007; Table 2). Spore production tended to increase following Cu-exposure by up to more than three-fold the control's value (Table 2). For S, in contrast, a slight trend to a decreased spore production was observed. The binary mixture caused at 500 µg Cu L^{-1} a significantly higher spore production compared to the control (Table 2). However, at 10 µg Cu L^{-1}, the increase in spore production was significantly lower compared to the single substance experiment with Cu-hydroxide (unpaired *t*-test; *P* = 0.030; Table 2).

DISCUSSION

LEAF PALATABILITY

The most remarkable result of the present study is that, contrary to our expectations, leaf palatability was positively affected by Cu-exposure, resulting in a preference of *Gammarus* for exposed leaf discs over control discs (Fig. 2a,b,c,e). Although this effect was detected irrespective of the tested Cu-compound, which is in line with our second hypothesis, it is in sharp contrast to the results of Tattersfield (1993). When exposing leaf material during the whole colonization period to Cu-sulfate, Tattersfield (1993) observed a negative effect on leaf palatability for *G. pulex*. However, in this study colonization phase lasted for only one week, which may be an insufficient time for a considerable colonization of the leaf material with microorganisms, in particular aquatic hyphomycetes (*cf*. Hieber and Gessner, 2002). Accordingly, when Tattersfield (1993) pre-conditioned leaves for one week prior to a seven-day Cu-exposure, indications for a positive effect on leaf palatability were observed, although these findings could not be explained mechanistically.

In this context, our study results indicate that the preference for Cu-exposed leaf material may be caused by the positive effect of Cu on leaf-associated fungal biomass (Fig. 4a,b,e), the most palatable component of decomposing leaf material for most shredders (as reviewed by Suberkropp, 1992). Since fungi and bacteria can act antagonistically on leaf material (Gulis and Suberkropp, 2003), the observed general trend to lower bacterial densities under Cu-exposure (Fig. 3a,b,c,e; Duarte et al., 2008; Duarte et al., 2009a) may have reduced the competitive pressure for fungi resulting in the higher fungal biomasses. This may also explain the observed trends to an increased fungal spore production (Roussel et al., 2008; Sridhar and Bärlocher, 2011) and a higher number of fungal species on Cu-exposed leaf discs (Table 2).

Likewise, Bundschuh *et al.* (2009) assumed the promoted fungal growth on antibiotics-exposed leaf material to be the result of negative effects on the bacterial community.

Another plausible explanation for the observed preference for Cu-exposed leaves may be the ability of *Gammarus* spp. to discriminate between different fungal species (Arsuffi and Suberkropp, 1989; Rong et al., 1995). Accordingly, the Cu-induced shift in aquatic hyphomycete community composition (Fig. S1) potentially triggered the altered leaf palatability for *Gammarus* (*cf*. Bundschuh et al., 2011). However, since spore production does not necessarily correlate with biomass (Bermingham et al., 1997), this inference should be treated with caution. To clarify the underlying mechanism, further developments in molecular biology such as quantitative real-time polymerase chain reaction (Fernandes et al., 2011), which would allow the estimation of single-species biomasses, may be beneficial.

Irrespective of the actual mechanism, the food-choice response of *Gammarus* indicates an altered food-quality of Cu-exposed leaf material. Zubrod *et al.* (2011) showed that the long-term consumption of organic fungicide-exposed leaf material, which was rejected in a food-choice experiment (Bundschuh et al., 2011), caused detrimental effects in the physiological fitness of *G. fossarum*. Conversely, gammarids may benefit from Cu-exposure via an improved food-quality, which may potentially counter the detrimental effects of direct Cu-toxicity in these animals when exposed to comparable concentrations (e.g. Zubrod et al., 2014).

MICROBIAL LEAF DECOMPOSITION

Aquatic hyphomycete species decompose leaves at different rates (Duarte et al., 2006). The change in fungal community composition (Fig. S1) may be one reason for the expected Cu-induced reduction in microbial leaf decomposition, with Cu-exposure potentially favoring less effective decomposers (Fig. 3a,b,c; see also Duarte et al., 2008; Duarte et al., 2009a; Pradhan et al., 2011; Tattersfield, 1993). Moreover, it was recently shown that fungal groups other than aquatic hyphomycetes are present on decomposing leaf material (Marano et al., 2011). Some of these (e.g. Chytridiomycota), however, do not produce ergosterol and were thus not considered during the determination of fungal biomass and community composition (the spore assessment exclusively allowed the identification of aquatic hyphomycetes). Therefore, negative effects on these groups and their potential contribution to decomposition cannot be ruled out. In any case, to further uncover the mechanism

behind the lower microbial decomposition induced by Cu, a deeper understanding of the microbial community composition and the contributions of single species to community functioning is needed. Also in this context, molecular biological methods could play a major role. This holds also true for communities exposed to S. While – against our expectations – this fungicide affected none of the assessed structural endpoints (Fig. 4d and S1; Table 2), it significantly reduced microbial decomposition (Fig. 3d), which cannot be explained by the mass of S potentially adsorbed to the leaf discs (Appendix S4).

JOINT EFFECT OF CU AND S

S did not have a significant effect when applied singly at 0.2 mg L^{-1} (Figs. 2d, 3d, 4d and S1; Table 2). Moreover, when S was jointly applied with Cu-hydroxide, Cu-concentrations realistic for agriculturally influenced surface waters (up to 263 µg L^{-1}; Süss et al., 2006) still resulted in effects on leaf-associated microbial communities (Fig. 4e; Table 2) and the functions they provide (Figs. 2e and 3e). However, in line with our fourth hypothesis, the binary mixture resulted in significantly lower effect sizes (Figs. 2a,e and 4a,e; Table 2) for nearly all assessed endpoints or even caused reversed effect directions (Fig. 3a,e; Table 2) compared to the single substance experiment with Cu-hydroxide. Thus, a true antagonistic mixture effect, as recently reported for the direct toxicity of a binary mixture of Cu and S on *G. fossarum* (Zubrod et al., 2014), seems plausible. In animals, S is known as dietary antagonist of Cu due to metabolic processes, *inter alia* sulfide formation, that make Cu biologically unavailable (e.g. Mills, 1985). Moreover, increased S-availability may also promote synthesis of thiol-containing compounds involved in Cu-detoxification (Guimarães-Soares et al., 2007) resulting in lower effect sizes than expected from the single substance experiment.

Although the use of separate controls (Table 1) for each fungicide (mixture) may be considered as a limitation of the present study, it allowed at the same time to control for most confounding factors. Nonetheless, all control aquatic hyphomycete communities except for the ones of the experiments with S and the binary mixture differed significantly from each other (PERMANOVA; $P \leq 0.03$). Moreover, bacterial densities in the controls varied among the experiments with a marginally non-significantly lower density for the binary mixture experiment compared to the experiment with Cu-hydroxide (Bonferroni-adjusted Wilcoxon rank-sum test; $P = 0.07$, Table S5). Consequently, the observed differences between the two

experiments could also be interpreted as a first hint that leaf-associated microbial communities are less sensitive towards Cu-stress during summer compared to winter. Therefore, further experimentation – controlling for potential season-related effects – seems necessary to allow final conclusions about the joint effect of Cu and S.

IMPLICATIONS FOR ENVIRONMENTAL RISK ASSESSMENT

Any effect on microorganisms involved in leaf litter breakdown may have far-reaching consequences for the detritus-based food web due to its bottom-up regulation (Wallace and Eggert, 1997; Zubrod et al., 2011). Environmental risk assessment for fungicides thus has to set threshold concentrations that are sufficiently protective to prevent such effects. Recently, HC_5-values (i.e. the hazardous concentration for 5% of species), derived from species sensitivity distributions, were suggested as protective benchmarks (Maltby et al., 2009). For Cu, reported HC_5-values range from 7.8 to 22.1 µg Cu L^{-1} depending on the physicochemical conditions (European Copper Institute, 2008). However, even the lower limit of this range is not protective for all endpoints assessed during the present study: the measured initial concentrations in the lowest treatment (nominal 10 µg Cu L^{-1}) of the experiments with Cu-hydroxide, Cu-sulfate and the binary mixture ranged from only 5.6 to 7.7 µg Cu L^{-1} (Table S1). Yet, these concentrations resulted in a reduced microbial leaf decomposition (Fig. 3c) and affected microbial community structure (e.g. fungal biomass; Fig. 4a,b,c and Table 2). Although an HC_5 for S could not be found in the literature, publicly available toxicity data (US EPA, 2014) indicate that existing threshold concentrations are in most cases much higher (up to the g L^{-1} range) than observed in this study (i.e. 0.8 mg L^{-1}). In light of the recent call by the EFSA (2013), microorganisms involved in leaf litter breakdown thus seem to be indeed adequate test organisms for the risk assessment of fungicides considering both their high sensitivity towards these substances and their crucial ecological role. Given the insufficient current state of knowledge regarding the sensitivity of single species and the biological interactions within leaf-associated microbial communities prone to chemical stress, the use of community-based test systems such as the one used during our study seems necessary to ensure an adequate level of protection. But community-based test systems are scheduled only if a risk was indicated by single-species standard toxicity tests (i.e. lower tier effect assessment; EFSA, 2013). However, as demonstrated above, these conventional standard toxicity tests seem to

be less sensitive than test systems including leaf-associated microbial communities and may thus fail to indicate a risk. This is further supported by an earlier study using the azole fungicide tebuconazole (Bundschuh et al., 2011), which resulted in structural and functional effects even at concentrations approximately five times lower than the respective HC_5 (*cf*. Maltby et al., 2009). Thus, standardization of community-based test systems for leaf-associated microorganisms and inclusion in lower tier risk assessment of fungicides seems to be the necessary starting point for a more protective fungicide risk assessment.

Moreover, our study indicates that Cu may affect aquatic bacteria and fungi as well as their functions at concentrations measured in surface waters adjacent to organic farming fields (up to 70 µg Cu L^{-1}; Süss et al., 2006). This suggests bottom-up effects in affected ecosystems and may explain the lack of deviations in leaf litter breakdown rates between streams influenced by conventional and organic farming (Magbanua et al., 2010). However, effects of organic agriculture on fundamental ecosystem functions are contrary to its underlying idea, namely to "respect nature's systems and cycles" (European Commission, 2007). In addition, elemental S, whose introduction in aquatic ecosystems has not been assessed in the past, may also be of concern considering its high authorized application rates (e.g. up to approximately 50 kg ha^{-1} a^{-1} in hop production in Germany; BVL, 2014) and the relatively low effect thresholds detected during this study. Therefore, when quantifying the ecological footprint of organic farming, the key ecosystem function of leaf litter breakdown should be considered.

In conclusion, we demonstrated that inorganic fungicide concentrations arising from organic and conventional farming may affect leaf-associated microorganisms, which justifies a careful reevaluation of the environmental safety of the agricultural use of these compounds. Moreover, our experimental design proved to be suitable for fungicide risk assessment purposes by uncovering effects of organic (Bundschuh et al., 2011) as well as inorganic fungicides (this study). Therefore, experimental designs similar to the one used during the present study may aid authorities in assessing the risk of (inorganic) fungicides for aquatic microorganisms more adequately with the ultimate purpose to safeguard the integrity of aquatic microbial communities and the functions they provide.

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APPENDIX S1 – MEASURED COPPER CONCENTRATIONS AND PHYSICOCHEMICAL CHARACTERISTICS OF CONDITIONING MEDIUM

Table S1. Nominal and mean measured (*n* = 3; with 95% CI) Cu-concentrations in fresh or 3-days old (collected prior to medium renewal) conditioning medium. Concentrations are given in µg Cu L^{-1}.

Experiment	10		500	
	Fresh	3-days old	Fresh	3-days old
Cu-hydroxide	5.8 (5.0-6.6)	1.4 (0.4-2.3)	351.8 (345.2-358.4)	8.3 (6.0-10.7)
Cu-octanoate	9.1 (5.0-13.2)	1.4 (1.0-1. 8)	491.7 (481.1-502.2)	7.2 (4.4-9.9)
Cu-sulfate	7.7 (7.4-8.0)	1.4 (0.9-1. 8)	452.0 (440.5-463.6)	7.3 (5.3-9.2)
Binary mixture	5. 6 (5.5-5. 7)	1.3 (0.8-1.8)	342.2 (331.9-352.4)	5. 6 (3.7-7.5)

Physicochemical characteristics of fresh and 3-days old (collected prior to the medium renewal) conditioning medium were measured in three control aquaria using a Multi 340i/SET (Wissenschaftlich- Technische-Werkstätten GmbH, Weilheim,

Germany) as well as visocolor® and nanocolor® kits (Macherey-Nagel, Düren, Germany).

Table S2. Mean (with 95% CI) physicochemical parameters of fresh and 3-days old (collected prior to medium renewal) conditioning medium.

Parameter	Fresh	3-days old
pH	7.01 (6.99-7.04)	7.04 (7.02-7.07)
Conductivity (µS cm^{-1})	346.0 (303.6-388.4)	334.0 (303.9-364.1)
Calcium (mg L^{-1})	33.3 (28.2-38.5)	29.8 (24.6-34.9)
Magnesium (mg L^{-1}) *	0 (0-0)	5.8 (0.0-12.0)
Chloride (mg L^{-1})	23.3 (9.0-37.7)	26.7 (12.3-41.0)
Sulfate (mg L^{-1})	<25 †	<25 †
Sulfide (mg L^{-1})	<0.05 †	<0.05 †
Nitrate (mg L^{-1})	21.7 (14.5-28.8)	26.7 (19.5-33.8)
Nitrite (mg L^{-1})	0.003 (0.000-0.007)	0.016 (0.012-0.020)
Ammonium (mg L^{-1})	<0.02 †	1.0 (0.1-1.8)
Ortho-phosphate (mg L^{-1})	2.0 (1.5-2.5)	2.4 (1.9-2.9)

* calculated as the difference between total hardness and calcium hardness

† below limit of quantification

APPENDIX S2 – FURTHER INFORMATION ABOUT THE AQUATIC HYPHOMYCETE COMMUNITIES IDENTIFIED DURING THIS STUDY

In total, the conidia of 38 species of aquatic hyphomycetes were identified during this study (Table S3), while only seven species accounted on average for 93% of all conidia in controls (mean contributions: *Alatospora acuminata* 13.3%, *Clavariopsis aquatica* 10.4%, *Flagellospora curvula* 7.5%, *Heliscella stellata* 10.0%, *Sigmoidea aurantiaca* 11.3%, *Tetracladium marchalianum* 30.8% and *Tricladium angulatum* 9.8%). Similarity percentage analysis (SIMPER; on square-root transformed data; Table S4) revealed that – with few exceptions – the seven dominant species also contributed for most of the dissimilarities between control and fungicide-exposed fungal communities (Fig. S1).

Table S3. List of all aquatic hyphomycete species identified during the experiments of the present study. Species in brackets were identified in only one sample of the experiment.

Species	Experiment				
	Cu-hydroxide	Cu-octanoate	Cu-sulfate	S	Binary mixture
Actinospora megalospora Ingold				(X)	
Alatospora acuminata Ingold	(X)	X	X	X	X
Anguillospora crassa Ingold				X	X
A. furtiva Descals					X
A. fustiformis Marvanová & Descals				X	X
A. longissima (Saccardo & P. Sydow) Ingold	X	X	X	X	X
Articulospora tetracladia Ingold		(X)	X		X
Camposporium pellucidum (Grove) S. Hughes	(X)		(X)	(X)	
Centrospora aquatica S.H. Iqbal	X	(X)		X	X
Clavariopsis aquatica De Wildeman	X	X	X	X	X
Clavatospora longibrachiata (Ingold) Sv. Nilsson ex Marvanová & Sv. Nilsson	X	X	X	X	X
Culicidospora gravida R.H. Petersen	(X)				
Dendrospora nana Descals & J. Webster	(X)				
Diplocladiella scalaroides G. Arnaud			(X)		
Flagellospora curvula Ingold	X	X	X	X	X
F. fusarioides S.H. Iqbal		(X)	(X)	X	
Geniculospora inflata (Ingold) Sv. Nilsson ex Marvanová & Sv. Nilsson				X	X
Heliscella stellata (Ingold & V.J. Cox) Marvanová		X	X	X	X
Lemonniera aquatica De Wildeman	X	X	X	X	
L. terrestris Tubaki	X	X	X	X	
Mycocentrospora clavata S.H. Iqbal					X
Pseudoanguillospora prolifera S.H. Iqbal				X	
P. stricta S.H. Iqbal				X	X
Sigmoidea aurantiaca Descals	X	X	X	X	X
Tetracladium furcatum Descals		X	X	(X)	
T. marchalianum De Wildeman	X	X	X	X	X
T. maxilliforme (Rostrup) Ingold			X		
T. setigerum (Grove) Ingold		X	X	X	
Tricladium angulatum Ingold	X	X	X	X	X
T. eccentricum R.H. Petersen				(X)	
T. gracile Ingold		X	(X)	X	
T. patulum Marvanová & Marvan					X
T. splendens Ingold				(X)	
T. terrestre D. Park		X	(X)	X	
Tripospermum camelopardus Ingold, Dann & P.J. McDougall			(X)		
T. myrti (Lind) S. Hughes				X	
T. prolongatum R.C. Sinclair & Morgan-Jones				(X)	
Volucrispora graminea Ingold, P.J. McDougall & Dann			(X)		
Total number (species detected only once)	14 (4)	18 (3)	22 (7)	28 (6)	18 (0)

Table S4. Results of SIMPER analysis, i.e. the five species contributing most to dissimilarities between fungicide treatments and the respective control.

Experiment	Concentration	Top 5 species with percentage contribution
Cu-hydroxide	10 µg Cu L^{-1}	*T. marchalianum* (28.0); *T. angulatum* (22.9); *S. aurantiaca* (14.6); *F. curvula* (10.7); *C. aquatica* (9.9)
	50 µg Cu L^{-1}	*T. angulatum* (25.4); *T. marchalianum* (21.0); *F. curvula* (16.6); *C. aquatica* (12.5); *S. aurantiaca* (11.8)
	250 µg Cu L^{-1}	*T. angulatum* (23.4); *T. marchalianum* (21.5); *S. aurantiaca* (17.9); *C. aquatica* (12.0); *F. curvula* (10.5)
	500 µg Cu L^{-1}	*T. angulatum* (31.1); *T. marchalianum* (20.2); *S. aurantiaca* (14.6); *C. aquatica* (9.1); *F. curvula* (7.5)
Cu-octanoate	10 µg Cu L^{-1}	*A. acuminata* (36.4); *T. marchalianum* (20.9); *C. aquatica* (7.6); *S. aurantiaca* (7.0); *T. terrestre* (5.1)
	50 µg Cu L^{-1}	*A. acuminata* (28.0); *T. marchalianum* (23.6); *C. aquatica* (12.9); *S. aurantiaca* (7.9); *T. terrestre* (5.9)
	250 µg Cu L^{-1}	*A. acuminata* (26.3); *T. marchalianum* (17.6); *C. aquatica* (10.1); *H. stellata* (8.2); *T. angulatum* (6.9)
	500 µg Cu L^{-1}	*A. acuminata* (20.1); *H. stellata* (17.3); *T. marchalianum* (14.8); *T. angulatum* (10.0); *T. terrestre* (7.3)
Cu-sulfate	10 µg Cu L^{-1}	*S. aurantiaca* (19.4); *T. marchalianum* (15.0); *C. aquatica* (14.6); *T. angulatum* (10.1); *A. longissima* (9.5)
	50 µg Cu L^{-1}	*T. marchalianum* (17.5); *C. aquatica* (16.0); *T. angulatum* (15.5); *S. aurantiaca* (13.5); *F. curvula* (8.7)
	250 µg Cu L^{-1}	*T. marchalianum* (22.4); *T. angulatum* (17.8); *C. aquatica* (13.9); *S. aurantiaca* (10.1); *H. stellata* (7.6)
	500 µg Cu L^{-1}	*T. marchalianum* (16.4); *C. aquatica* (15.0); *T. angulatum* (13.2); *H. stellata* (9.6); *S. aurantiaca* (8.0)
S	0.05 mg L^{-1}	*T. marchalianum* (21.0); *H. stellata* (18.1); *T. angulatum* (12.8); *A. acuminata* (12.7); *C. longibrachiata* (10.5)
	0.2 mg L^{-1}	*T. marchalianum* (19.8); *H. stellata* (18.5); *A. acuminata* (12.7); *T. angulatum* (12.6); *C. longibrachiata* (10.0)
	0.8 mg L^{-1}	*T. marchalianum* (23.2); *H. stellata* (14.2); *T. angulatum* (13.4); *A. acuminata* (13.1); *C. longibrachiata* (8.4)
	3.2 mg L^{-1}	*T. marchalianum* (21.7); *H. stellata* (19.6); *T. angulatum* (12.8); *A. acuminata* (12.1); *C. longibrachiata* (8.0)
Binary mixture	10 µg Cu L^{-1}	*T. marchalianum* (24.4); *H. stellata* (20.8); *C. longibrachiata* (11.7); *T. angulatum* (10.7); *S. aurantiaca* 7.3)
	50 µg Cu L^{-1}	*T. marchalianum* (24.3); *H. stellata* (19.0); *C. longibrachiata* (12.6); *T. angulatum* (10.9); *S. aurantiaca* (10.1)
	250 µg Cu L^{-1}	*H. stellata* (23.0); *T. marchalianum* (20.2); *T. angulatum* (15.3); *C. longibrachiata* (11.9); *C. aquatica* (7.7)
	500 µg Cu L^{-1}	*H. stellata* (37.1); *T. angulatum* (13.5); *C. longibrachiata* (13.3); *T. marchalianum* (13.2); *S. aurantiaca* (6.3)

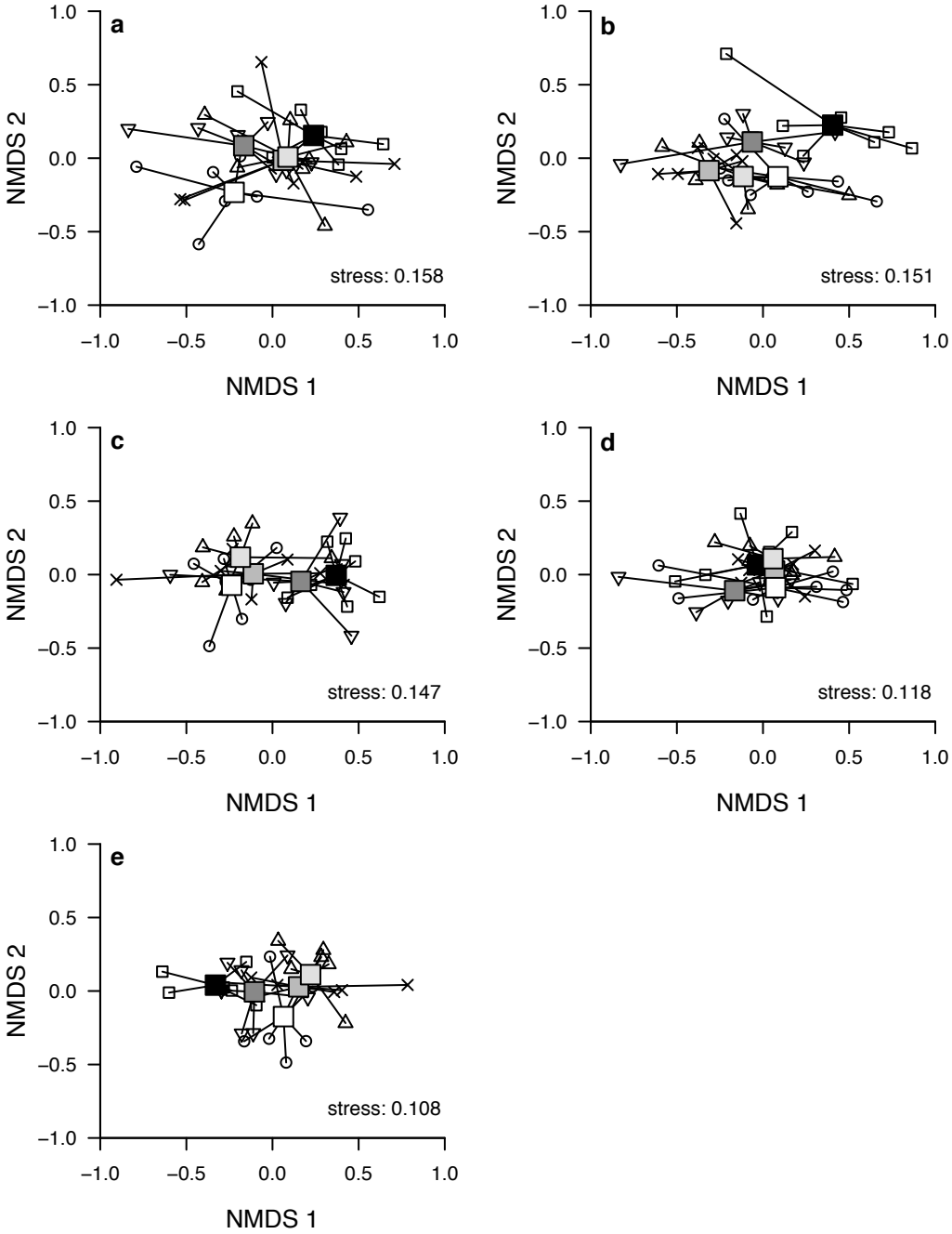

Fig. S1. Non-metric multidimensional scaling (NMDS) plots of aquatic hyphomycete communities associated with leaf material conditioned in the presence of different concentrations of a) Cu-hydroxide, b) Cu-octanoate, c) Cu-sulfate, d) S and e) Cu-hydroxide amended by 0.2 mg S L^{-1}. Symbols are (from lowest to highest concentration): circles, triangles, crosses, inverted triangles and squares. Additionally, concentrations are indicated by grey scale of the group centroids with white and black corresponding to the lowest (=control) and the highest tested concentration, respectively. Stress values were calculated as a measure of "goodness-of-fit" with values <0.2 representing reasonable fits (Clarke, 1993).

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APPENDIX S3 – GROUP MEANS OR MEDIANS AND CORRESPONDING 95% CIS ON THE ORIGINAL SCALE

Average total leaf consumption (i.e. the sum of consumption on the control leaf disc and the fungicide-exposed disc) of *G. fossarum* ranged from 0.2 to 0.5 mg mg^{-1} day^{-1} (Table S5). These values match well with feeding rates of *Gammarus* measured in the laboratory (e.g. Zubrod *et al.* 2014) and in the field (Maltby et al., 2002; Bundschuh et al., 2011).

Average microbial decomposition in the controls corresponded to approximately 60% of initial leaf mass (data not shown), which is comparably high for black alder conditioned for 13 days (e.g. Hieber and Gessner, 2002). This may be explained by the rather high temperature and nutrient supply during conditioning (*cf*. Ferreira and Chauvet, 2011) as well as the absence of main veins in the leaf discs and the physical destruction of cell structures caused by cutting the leaves.

Bacteria counts depend strongly on the method used for detaching bacteria from the leaf surface and the used dye (*cf*. Weyers and Suberkropp, 1996). Thus, only comparisons with studies using the same combination of detachment technique and dye seem valid. The average control density of 6.2×10^8 cells mg^{-1} (Table S5) observed in the present study compares well with these studies (e.g. Zubrod et al., 2011).

Average fungal biomass associated with control leaf discs was approximately 270 µg ergosterol g^{-1} when excluding the exceptionally high value measured in the Cu-sulfate experiment (see main body of manuscript for reasoning; Table S5), which corresponds to approximately 50 mg fungal biomass g^{-1} (Gessner and Chauvet, 1993). This is well within the range of reported fungal biomasses associated with black alder in the field (e.g. up to 80 mg g^{-1}; Hieber and Gessner, 2002). Compared to the field, however, fungal biomass accrual in the present study was relatively fast, probably also driven by the same reasons as suggested for the fast microbial decomposition.

Table S5. Group means or medians (with 95% CI) on the original scale.

Fungicde	Endpoint	Concentration	Mean or median	95% CI
Cu-hydroxide	Leaf consumption in mg mg^{-1} day^{-1}	0 µg Cu L^{-1} (for 10 µg Cu L^{-1})	0.11	0.04-0.18
		10 µg Cu L^{-1}	0.14	0.07-0.21
		0 µg Cu L^{-1} (for 50 µg Cu L^{-1})	0.19	0.09-0.29
		50 µg Cu L^{-1}	0.21	0.12-0.30
		0 µg Cu L^{-1} (for 250 µg Cu L^{-1})	0.12	0.03-0.20
		250 µg Cu L^{-1}	0.23	0.15-0.32
		0 µg Cu L^{-1} (for 500 µg Cu L^{-1})	0.03	0.00-0.09
		500 µg Cu L^{-1}	0.17	0.07-0.26
	Microbial decomposition in mg day^{-1}	0 µg Cu L^{-1} (for 10 µg Cu L^{-1})	0.18	0.17-0.20
		10 µg Cu L^{-1}	0.17	0.16-0.19
		0 µg Cu L^{-1} (for 50 µg Cu L^{-1})	0.21	0.19-0.23
		50 µg Cu L^{-1}	0.22	0.20-0.24
		0 µg Cu L^{-1} (for 250 µg Cu L^{-1})	0.24	0.23-0.30
		250 µg Cu L^{-1}	0.23	0.22-0.23
		0 µg Cu L^{-1} (for 500 µg Cu L^{-1})	0.27	0.22-0.30
		500 µg Cu L^{-1}	0.22	0.19-0.23
	Bacterial density in 10^8 cells mg^{-1}	0 µg Cu L^{-1}	5.2	3.6-6.8
		10 µg Cu L^{-1}	5.2	4.3-6.1
		50 µg Cu L^{-1}	4.1	3.1-5.1
		250 µg Cu L^{-1}	4.0	3.2-4.7
		500 µg Cu L^{-1}	3.7	2.9-4.6
	Fungal biomass in mg ergosterol g^{-1}	0 µg Cu L^{-1}	244.6	221.6-289.7
		10 µg Cu L^{-1}	331.7	250.7-450.0
		50 µg Cu L^{-1}	346.7	264.8-352.2
		250 µg Cu L^{-1}	400.3	288.7-421.2
		500 µg Cu L^{-1}	499.0	335.5-571.8
Cu-octanoate	Leaf consumption in mg mg^{-1} day^{-1}	0 µg Cu L^{-1} (for 10 µg Cu L^{-1})	0.13	0.08-0.17
		10 µg Cu L^{-1}	0.13	0.08-0.18
		0 µg Cu L^{-1} (for 50 µg Cu L^{-1})	0.14	0.10-0.18
		50 µg Cu L^{-1}	0.10	0.06-0.14
		0 µg Cu L^{-1} (for 250 µg Cu L^{-1})	0.11	0.06-0.16
		250 µg Cu L^{-1}	0.16	0.11-0.22
		0 µg Cu L^{-1} (for 500 µg Cu L^{-1})	0.18	0.11-0.24
		500 µg Cu L^{-1}	0.20	0.12-0.27
	Microbial decomposition in mg day^{-1}	0 µg Cu L^{-1} (for 10 µg Cu L^{-1})	0.25	0.24-0.26
		10 µg Cu L^{-1}	0.24	0.23-0.25
		0 µg Cu L^{-1} (for 50 µg Cu L^{-1})	0.24	0.23-0.25
		50 µg Cu L^{-1}	0.24	0.23-0.25
		0 µg Cu L^{-1} (for 250 µg Cu L^{-1})	0.20	0.19-0.21
		250 µg Cu L^{-1}	0.19	0.17-0.20
		0 µg Cu L^{-1} (for 500 µg Cu L^{-1})	0.21	0.20-0.23
		500 µg Cu L^{-1}	0.18	0.16-0.19

Table S5 continued.

Fungicde	Endpoint	Concentration	Mean or median	95% CI
Cu-octanoate	Bacterial density	0 µg Cu L^{-1}	12.0	3.8-20.3
	in 10^8 cells mg^{-1}	10 µg Cu L^{-1}	5.6	4.8-6.4
		50 µg Cu L^{-1}	5.8	4.0-7.5
		250 µg Cu L^{-1}	6.2	5.5-7.0
		500 µg Cu L^{-1}	5.3	4.1-6.5
	Fungal biomass	0 µg Cu L^{-1}	274.6	184.6-364.6
	in mg ergosterol g^{-1}	10 µg Cu L^{-1}	313.2	212.1-414.3
		50 µg Cu L^{-1}	330.2	296.6-363.8
		250 µg Cu L^{-1}	421.0	326.6-515.4
		500 µg Cu L^{-1}	487.7	378.0-597.4
Cu-sulfate	Leaf consumption	0 µg Cu L^{-1} (for 10 µg Cu L^{-1})	0.26	0.12-0.40
	in mg mg^{-1} day^{-1}	10 µg Cu L^{-1}	0.19	0.06-0.31
		0 µg Cu L^{-1} (for 50 µg Cu L^{-1})	0.23	0.15-0.32
		50 µg Cu L^{-1}	0.18	0.08-0.29
		0 µg Cu L^{-1} (for 250 µg Cu L^{-1})	0.24	0.11-0.37
		250 µg Cu L^{-1}	0.28	0.14-0.42
		0 µg Cu L^{-1} (for 500 µg Cu L^{-1})	0.09	0.00-0.23
		500 µg Cu L^{-1}	0.33	0.18-0.48
	Microbial decomposition	0 µg Cu L^{-1} (for 10 µg Cu L^{-1})	0.36	0.31-0.41
	in mg day^{-1}	10 µg Cu L^{-1}	0.27	0.24-0.30
		0 µg Cu L^{-1} (for 50 µg Cu L^{-1})	0.40	0.37-0.44
		50 µg Cu L^{-1}	0.35	0.32-0.38
		0 µg Cu L^{-1} (for 250 µg Cu L^{-1})	0.37	0.34-0.40
		250 µg Cu L^{-1}	0.27	0.24-0.29
		0 µg Cu L^{-1} (for 500 µg Cu L^{-1})	0.35	0.30-0.39
		500 µg Cu L^{-1}	0.24	0.21-0.26
	Bacterial density	0 µg Cu L^{-1}	8.4	2.3-14.5
	in 10^8 cells mg^{-1}	10 µg Cu L^{-1}	3.3	1.9-4.7
		50 µg Cu L^{-1}	4.9	2.3-7.5
		250 µg Cu L^{-1}	4.2	2.7-5.7
		500 µg Cu L^{-1}	5.1	3.5-6.8
	Fungal biomass	0 µg Cu L^{-1}	426.7	359.4-494.0
	in mg ergosterol g^{-1}	10 µg Cu L^{-1}	449.2	354.5-543.9
		50 µg Cu L^{-1}	382.1	264.0-500.2
		250 µg Cu L^{-1}	344.5	245.7-443.4
		500 µg Cu L^{-1}	390.9	343.5-438.3
Sulfur	Leaf consumption	0 mg L^{-1} (for 0.05 mg L^{-1})	0.10	0.05-0.17
	in mg mg^{-1} day^{-1}	0.05 mg L^{-1}	0.10	0.03-0.14
		0 mg L^{-1} (for 0.2 mg L^{-1})	0.14	0.04-0.24
		0.2 mg L^{-1}	0.12	0.04-0.20
		0 mg L^{-1} (for 0.8 mg L^{-1})	0.18	0.11-0.32
		0.8 mg L^{-1}	0.22	0.15-0.37
		0 mg L^{-1} (for 3.2 mg L^{-1})	0.11	0.04-0.20
		3.2 mg L^{-1}	0.19	0.11-0.30

Table S5 continued.

Fungicde	Endpoint	Concentration	Mean or median	95% CI
	Microbial decomposition in mg day^{-1}	0 mg L^{-1} (for 0.05 mg L^{-1})	0.15	0.13-0.17
		0.05 mg L^{-1}	0.15	0.13-0.18
		0 mg L^{-1} (for 0.2 mg L^{-1})	0.17	0.15-0.20
		0.2 mg L^{-1}	0.18	0.16-0.19
		0 mg L^{-1} (for 0.8 mg L^{-1})	0.14	0.13-0.15
		0.8 mg L^{-1}	0.13	0.11-0.13
		0 mg L^{-1} (for 3.2 mg L^{-1})	0.22	0.20-0.25
		3.2 mg L^{-1}	0.19	0.17-0.21
	Bacterial density in 10^8 cells mg^{-1}	0 mg L^{-1}	2.7	1.6-9.9
		0.05 mg L^{-1}	2.2	1.8-8.8
		0.2 mg L^{-1}	2.4	1.6-2.7
		0.8 mg L^{-1}	2.7	1.7-11.9
		3.2 mg L^{-1}	2.2	0.9-5.2
	Fungal biomass in mg ergosterol g^{-1}	0 mg L^{-1}	264.8	189.1-320.5
		0.05 mg L^{-1}	260.8	215.1-313.8
		0.2 mg L^{-1}	289.3	239.8-415.8
		0.8 mg L^{-1}	278.6	151.4-336.0
		3.2 mg L^{-1}	251.2	220.3-294.2
Binary mixture	Leaf consumption in mg mg^{-1} day^{-1}	0 µg Cu L^{-1} (for 10 µg Cu L^{-1})	0.15	0.11-0.19
		10 µg Cu L^{-1}	0.13	0.08-0.19
		0 µg Cu L^{-1} (for 50 µg Cu L^{-1})	0.17	0.11-0.24
		50 µg Cu L^{-1}	0.17	0.12-0.23
		0 µg Cu L^{-1} (for 250 µg Cu L^{-1})	0.21	0.15-0.27
		250 µg Cu L^{-1}	0.17	0.11-0.23
		0 µg Cu L^{-1} (for 500 µg Cu L^{-1})	0.19	0.14-0.25
		500 µg Cu L^{-1}	0.31	0.23-0.39
	Microbial decomposition in mg day^{-1}	0 µg Cu L^{-1} (for 10 µg Cu L^{-1})	0.16	0.15-0.17
		10 µg Cu L^{-1}	0.15	0.15-0.16
		0 µg Cu L^{-1} (for 50 µg Cu L^{-1})	0.16	0.15-0.17
		50 µg Cu L^{-1}	0.16	0.15-0.17
		0 µg Cu L^{-1} (for 250 µg Cu L^{-1})	0.17	0.16-0.18
		250 µg Cu L^{-1}	0.18	0.17-0.19
		0 µg Cu L^{-1} (for 500 µg Cu L^{-1})	0.28	0.26-0.29
		500 µg Cu L^{-1}	0.28	0.27-0.29
	Bacterial density in 10^8 cells mg^{-1}	0 µg Cu L^{-1}	2.5	1.7-3.2
		10 µg Cu L^{-1}	2.4	1.7-3.1
		50 µg Cu L^{-1}	2.4	1.8-2.9
		250 µg Cu L^{-1}	2.5	2.1-3.0
		500 µg Cu L^{-1}	2.1	2.0-2.3
	Fungal biomass in mg ergosterol g^{-1}	0 µg Cu L^{-1}	279.3	246.5-312.1
		10 µg Cu L^{-1}	279.2	259.5-298.9
		50 µg Cu L^{-1}	298.7	277.0-320.4
		250 µg Cu L^{-1}	368.9	325.6-412.2
		500 µg Cu L^{-1}	450.9	387.8-514.1

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APPENDIX S4 – MASS OF SULFUR POTENTIALLY ADSORBED TO LEAF DISCS

Elemental sulfur (S) was applied at rather high concentrations during this study (up to 3.2 mg L^{-1}) and is moderately affine to organic material (organic carbon/water partitioning coefficient = 1,950; FOOTPRINT, 2013). One may thus assume that the effect sizes observed for S-affected microbial leaf decomposition (~10%; Fig. 3d) may in fact be an erroneously interpreted artifact from adsorption of the substance to the leaf discs. However, the following calculations show that this may explain only a fraction of the observed effect sizes:

At 0.8 and 3.2 mg L^{-1}, i.e. the concentrations which resulted in significant effects on decomposition, the total mass of S applied to one aquarium was (nominal concentration x 4 L x 4 medium renewals) 12.8 and 51.2 mg, respectively. One aquarium contained 68 leaf discs with a leaching-corrected mean dry weight of 7.0 mg (6.9-7.2; 95% CI) prior to conditioning, estimated from the pre-weighed discs for the food-choice experiment. This results in a total dry weight of 476 mg. In addition to the leaf discs, also inoculum (10 g fresh weight) was present in the aquaria. Three 10.0 g portions of inoculum leaves were dried for 24 h before being weighed again and a mean dry weight of 1.8 g (1.5-2.1; 95% CI) was obtained, which accounts for approximately 80% of the leaf mass present in the aquaria. Assuming that S has the same affinity for the unconditioned leaf discs and the inoculum, approximately 20% of the whole amount of S might adsorb to the leaf discs' surfaces (i.e. 2.7 and 10.8 mg in total or 0.04 and 0.16 mg per disc, respectively). The median differences in microbial leaf decomposition between the control and the two S-treatments were 0.24 and 0.32 mg. Thus, even if all S actually adsorbed to the leaf material in the aquaria, only about 17% (at 0.8 mg L^{-1}) and 50% (3.2 mg L^{-1}) of the observed effect sizes could be explained by the mass of S. If corrected for this potential but, at the calculated size, unlikely bias, microbial decomposition would still be statistically significantly reduced at 0.8 mg L^{-1} (median reduction 9.3%, Wilcoxon signed-rank test, $P < 0.001$), while at 3.2 mg L^{-1} still a tendency to such an effect could be observed (median reduction 5.3%, Wilcoxon signed-rank test, $P = 0.17$).

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APPENDIX A.3

Does the current fungicide risk assessment provide sufficient protection for key drivers in aquatic ecosystem functioning?

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ABSTRACT

The level of protection provided by the present environmental risk assessment (ERA) of fungicides in the European Union for fungi is unknown. Therefore, we assessed the structural and functional implications of five fungicides with different modes of action (azoxystrobin, carbendazim, cyprodinil, quinoxyfen, and tebuconazole) individually and in mixture on communities of aquatic hyphomycetes. This is a polyphyletic group of fungi containing key drivers in the breakdown of leaf litter, governing both microbial leaf decomposition and the palatability of leaves for leaf-shredding macroinvertebrates. All fungicides impaired leaf palatability to the leaf-shredder *Gammarus fossarum* and caused structural changes in fungal communities. In addition, all compounds except for quinoxyfen altered microbial leaf decomposition. Our results suggest that the European Union's first-tier ERA provides sufficient protection for the tested fungicides, with the exception of tebuconazole and the mixture, while higher-tier ERA does not provide an adequate level of protection for fungicides in general. Therefore, our results show the need to incorporate aquatic fungi as well as their functions into ERA testing schemes to safeguard the integrity of aquatic ecosystems.

INTRODUCTION

Fungal diseases are among the major threats for crop production (Fisher et al., 2012), rendering the application of fungicides essential to secure global food supply (Strange and Scott, 2005). Following their application, these substances can enter surface water bodies via point (e.g., wastewater treatment plant outlets) and diffuse (e.g., surface run-off) sources (Bereswill et al., 2012; Kahle et al., 2008), potentially posing a risk to the integrity of aquatic ecosystems in general and their inhabiting fungi in particular. However, little is known about the sensitivity of non-target fungal communities towards fungicides and, in consequence, about the level of protection of the European Union's environmental risk assessment (ERA) for these organisms and the ecosystem functions they provide (EFSA, 2013; Maltby et al., 2009).

To assess this, aquatic hyphomycetes (i.e., a polyphyletic group of aquatic fungi), being of paramount importance for the breakdown of allochthonous organic matter (particularly leaf litter; Gessner et al., 2007), seem to be a suitable starting point. In the process of organic matter breakdown, aquatic hyphomycetes accomplish two important functions: first, they can be the major drivers of biotic leaf mass loss and thus for the incorporation of the energy bound in leaves into stream food webs

(Gessner et al., 2007; Taylor and Chauvet, 2014). Second, they transform leaves chemically and physically (i.e., conditioning), thereby increasing their palatability and nutritional value for leaf-shredding macroinvertebrates (Bärlocher, 1985). However, there are no comprehensive studies targeting effects of individual synthetic fungicides and their mixtures on both structural and functional endpoints in aquatic hyphomycetes (but see Zubrod et al., 2015 for inorganic fungicides), while several studies on single demethylation-inhibiting fungicides (Artigas et al., 2012; Bundschuh et al., 2011a; Dimitrov et al., 2014; Flores et al., 2014; Zubrod et al., 2011) suggest low effect threshold concentrations.

In this context, we empirically assessed whether current fungicide ERA practices in the European Union provide an acceptable level of protection for aquatic hyphomycetes using five current-use fungicides with different modes of toxic action (Tab. 1). These fungicides were investigated singly and in combination. The latter approach was prompted by the frequent detection of fungicides in complex mixtures in the field (Bereswill et al., 2012). Our experimental design allowed assessing the two important functions provided by aquatic hyphomycetes: i.e., microbial leaf decomposition as well as leaf palatability, which was studied via the food-choice of the highly selective amphipod *Gammarus fossarum* KOCH (Arsuffi and Suberkropp, 1989), a key shredder in many European low order streams (Englert et al., 2013). Moreover, to gain a mechanistic understanding of potential implications at the functional level, the leaf-associated microbial communities were characterized. We expected the fungicides to have negative effects on both aquatic hyphomycete community structure and functioning (Artigas et al., 2012; Bundschuh et al., 2011a; Dimitrov et al., 2014; Flores et al., 2014; Zubrod et al., 2011), while their mixture effect was hypothesized to meet or exceed reference model predictions as shown for the direct toxicity (i.e., exposure via water phase) of the same fungicide mixture towards *G. fossarum* (Zubrod et al., 2014).

MATERIAL AND METHODS

CHEMICALS

For the five single-substance experiments, nominal concentrations of the tested fungicide active ingredients (applied as products; Table 1) were selected considering effect concentrations reported for aquatic hyphomycetes and other aquatic fungi (Artigas et al., 2012; Bundschuh et al., 2011a; Dijksterhuis et al., 2011; Dimitrov et al., 2014; Flores et al., 2014; Zubrod et al., 2011), aiming to observe a gradient from

little to strong effects on leaf-associated fungal communities' structure and functioning. The mixture experiment employed a fixed-concentration-ratio with the total fungicide concentrations covering approximately those of the single-substance experiments (i.e., 1, 10, 100, and 500 µg/L for each substance; Tables 1 and S1). The only exception was quinoxyfen, which was applied at concentrations twice as high as the remaining substances (i.e., 2, 20, 200, and 1,000 µg/L; Tables 1 and S1) to counteract its fast dissipation from the water phase (Table S2). For the preparation of stock solutions and subsequent serial dilution, a nutrient medium (i.e., conditioning medium; for composition see Table S3) was used (Dang et al., 2005). Shortly after fungicide application (i.e., after thorough mixing but before introduction of leaf material) and at the time of the first medium renewal (i.e., after three days; see below), triplicate 10-mL samples of the treatments with the lowest and the highest tested concentrations as well as one sample from the respective controls were frozen (-20°C) until further processing. Fungicide concentrations were verified using ultra-high performance liquid chromatography-mass spectrometry (Thermo Fisher Scientific).(Zubrod et al., 2011) Since measured initial concentrations and nominal ones agreed well (Table S2), the latter are reported throughout this paper. Unless otherwise specified, chemicals were purchased from Sigma-Aldrich or Roth.

Table 1. Products, producers, modes of action, and nominal concentrations of the fungicides.

Experiment	Product	Producer	Mode of action(FRAC, 2015)	Nominal concentrations (µg/L)
Azoxystrobin	Ortiva	Syngenta Agro	Inhibition of mitochondrial respiration	20; 100; 500; 2,500
Carbendazim	Derosal	Bayer CropScience	Inhibition of mitosis and cell division	5; 35; 245; 1,715
Cyprodinil	Chorus	Syngenta Agro	Inhibition of amino acid and protein synthesis	8; 40; 200; 1,000
Quinoxyfen	Fortress 250	Dow AgroSciences	Perturbation of signal transduction	5; 40; 320; 2,560
Tebuconazole	Folicur	Bayer CropScience	Inhibition of sterol biosynthesis	1; 5; 50; 500
Mixture	All of the above	All of the above	All of the above	6; 60; 600; 3,000

SOURCES OF LEAVES, MICROORGANISMS, AND GAMMARIDS

Experimental procedures are described in more detail in Zubrod et al. (2015). Briefly, *Alnus glutinosa* (L.) GAERTN. (black alder) leaves were collected in October 2011 (near Landau, Germany; 49°11'N, 8°05'E) and stored at -20°C until further use. For each of the six experiments, 500 alder leaves were deployed in fine-mesh bags (0.5 mm mesh size; 10 leaves per bag) in the Rodenbach near Grünstadt (Germany;

49°33'N, 8°02'E) upstream of any agricultural activity, settlement, and wastewater inlet for 14 days. Back in the laboratory, another 500 leaves were added and leaves were mixed and kept for a further 14 days in 30 L of aerated conditioning medium at 16±1°C in total darkness (complete medium renewal after seven days) before being used as microbial inoculum in the experiments (see below). This procedure ensured inoculum with leaves featuring microbial communities at different successional stages and thus presumably a high diversity of aquatic hyphomycetes (Gessner et al., 1993).

In the Hainbach near Frankweiler (Germany; 49°14'N, 8°03'E; upstream of any agricultural activity, settlement, and wastewater inlet), *G. fossarum* (cryptic lineage B; Feckler et al., 2014) were kick-sampled seven days prior to their use in experiments. Only adult males of approximately 6 to 8 mm body length being visually free from acanthocephalan parasites were used. During their acclimation (20±1°C, total darkness), gammarids were gradually adapted to amphipod medium (i.e., SAM-5S; Borgmann, 1996; for composition see Table S3). For the first three days in the laboratory, amphipods received pre-conditioned black alder leaves *ad libitum*. Afterwards, they were starved to level their appetite.

MAIN EXPERIMENTS ON FUNCTIONAL AND STRUCTURAL EFFECTS

Each experiment comprised four fungicide concentrations plus a fungicide-free control. To assess both functional endpoints (i.e., microbial leaf decomposition and gammarids' food choice), sets of four leaf discs (diameter = 16 mm) were cut out of unconditioned leaves. The discs were dried at 60°C for 24 h and weighed individually to the nearest 0.01 mg. Subsequently, two discs per leaf were conditioned in the fungicide-free control, while the remaining two were exposed to one of the fungicide concentrations. For this purpose, after sewing them individually into labeled and thoroughly leached fiber glass gaze bands, the discs were placed in 5-L round glass aquaria (seven for each of the five fungicide concentrations) together with additional discs for the assessment of structural endpoints (see below). Each aquarium was filled with 4 L of conditioning medium amended with the respective fungicide concentration(s) and received ten grams (wet weight after blotting) of inoculum. Aquaria were kept at 16±1°C in total darkness and were continuously stirred and aerated. Test solutions were renewed every three days to ensure a continuous fungicide exposure over the 12-days conditioning period. This chronic exposure without pre-conditioning of leaf discs in fungicide-free medium was considered the

realistic worst-case scenario given the continuous presence of fungicides in agricultural streams (Reilly et al., 2012).

After twelve days, the leaf discs intended for assessing the functional endpoints were rinsed in fungicide-free SAM-5S for 30 min. Two leaf discs originating from the same leaf (i.e., one conditioned in the control and one conditioned in the presence of fungicide(s)) were fixed in the center of a food-choice arena (= 300-mL crystallization dish) filled with 100 mL fungicide-free SAM-5S (Bundschuh et al., 2009). Subsequently, one gammarid was allowed to feed on the two discs for 24 h (at 20±1°C in total darkness). In the same arena, the two corresponding discs from the same leaf were protected from amphipod feeding by 0.5-mm fiberglass mesh screen. These discs were used to quantify the microbial leaf decomposition and abiotic mass losses during the experiments. Additionally, this procedure allowed for controlling for these factors when calculating gammarids' leaf consumption. After 24 h, the gammarids (49 per fungicide concentration) as well as any remaining leaf material were dried and weighed as described above. Replicates containing animals that died or molted during the experiments were excluded from evaluation of the food-choice data.

SUPPLEMENTAL STUDY ON THE REPELLENT EFFECT OF FUNGICIDES

To assess the potential impact of a repellent effect caused by adsorbed fungicides (*cf.* Hahn & Schulz, 2007; Rasmussen et al., 2012), additional experiments were performed that excluded any fungicide-induced changes in the leaf associated microbial communities. Therefore, pre-conditioned leaf discs (Bundschuh et al., 2011b) were weighed, autoclaved and exposed to each of the individual fungicides and the mixture in 4 L of autoclaved conditioning medium containing 10 g (wet weight after blotting) of autoclaved microbial inoculum simulating the procedure detailed above. While one aquarium contained no fungicides (i.e., control), the second one contained the respective fungicide or the mixture at an aqueous-phase concentration four-times higher than the lowest concentration triggering a significant food-choice response in the main experiments (or four-fold the highest concentration if no significant effect was found). This procedure was selected to account for the total amount of fungicide applied during microbial conditioning in the main experiments (note that the medium was renewed four times) that potentially adsorbed to the leaf discs' surfaces. After three days, leaf discs from the control and from the fungicide treatment were rinsed and simultaneously offered to one gammarid for 24 h (n = 49).

CHARACTERIZATION OF LEAF-ASSOCIATED MICROBIAL COMMUNITIES

Microbial communities were characterized on leaf discs conditioned together with the discs used for assessing the functional endpoints during the main experiments resulting in seven independent replicates per treatment. Aquatic hyphomycete community composition was assessed via spore morphology generally following Bärlocher (1982). Leaf discs were agitated in deionized water for 96 h at 20°C before fixing and staining fungal spores by adding lactophenol cotton blue. Spores were identified under a microscope using several identification keys (e.g., Ingold, 1975).

Ergosterol was quantified as proxy for leaf-associated fungal biomass according to Gessner (2005). After extraction in alkaline methanol, ergosterol was purified by solid-phase extraction (Sep-Pak® Vac RC tC18 500 mg sorbent, Waters), and quantified by high-performance liquid chromatography (1200 Series, Agilent Technologies).

Since leaf-associated bacteria can interact with aquatic hyphomycetes (Gulis and Suberkropp, 2003), bacterial densities (number of cells per mg leaf) were determined according to Buesing (2005). Dislodged bacteria were stained using SYBRGreen II (Molecular Probes). Subsequently, digital pictures were taken using an epifluorescence microscope and cells were counted by image analysis software (Axio Scope.A1, AxioCam MRm, and AxioVision, Carl Zeiss MicroImaging).

STATISTICAL ANALYSES

Microbial leaf decomposition (D) was calculated as $D = (n_b*0.74-n_a)/t$ (Zubrod et al., 2015). There, n_b and n_a refer to the initial and final dry weight of the leaf discs protected from amphipod feeding, respectively, 0.74 is an empirical factor controlling for leaching of the leaf material, and t is decomposition time (i.e., 13 days). Gammarids' leaf consumption (C) was calculated as $C = [(f_b-f_a)-(n_b-n_a)]/(g*t)$ and $C = (f_b*k-f_a)/(g*t)$ for the main and supplemental food-choice experiments, respectively.(Bundschuh et al., 2009; Bundschuh et al., 2011b) There, f_b is the initial dry weight of the leaf disc accessible for the gammarid and f_a is the dry weight of the same disc after the food choice experiment. The gammarid's dry weight is indicated by g and feeding time (t) was one day. The leaf weight correction factor k, correcting for abiotic mass losses during the supplementary experiments, was calculated experiment-wise as $k = \sum(1-(n_b-n_a)/n_b)/10$ using the initial (n_b) and final (n_a) dry weight of ten leaf discs not accessible for gammarids.

Univariate data were checked for normality by visual inspection, while homoscedasticity was tested via Levene's test. To assess statistically significant differences between the fungicide treatments and the corresponding controls for microbial leaf decomposition and gammarids' leaf consumption, paired *t*-tests or, as a non-parametric alternative, Wilcoxon signed-rank tests were used (note the paired design according to the criteria for instance described by Zar, 2010). For the remaining univariate data (for instance fungal biomass and bacterial density), fungicide treatments were compared to the controls by performing ANOVAs followed by Dunnett's tests or – if the assumptions for parametric testing were not met – Kruskal-Wallis tests followed by Wilcoxon rank-sum tests using the Bonferroni adjustment for multiple comparisons (for all univariate analyses see Zar, 2010).

To assess shifts in aquatic hyphomycete community composition, permutational multivariate analysis of variance (PERMANOVA) was used, which is a non-parametric, permutation-based procedure to assess for statistically significant differences between groups of multivariate data (Clarke and Warwick, 2001). Analyses were performed on Bray-Curtis dissimilarities, while data were square-root transformed to reduce the effect of dominant species (Clarke and Warwick, 2001). Species determined in only one sample of an experiment were excluded to reduce arbitrary noise. In the case of samples without fungal spores, which was mostly related to fungicide treatment, a "dummy species" with an abundance of one was added to each sample of the respective experiment since Bray-Curtis dissimilarity is undefined for empty samples (Clarke et al., 2006). Results of additional analyses and visualizations of the aquatic hyphomycete communities are provided in the Supporting Information.

To assess the joint effects of the five fungicides during the mixture experiment, observed effect sizes were compared to predictions by a reference model for mixtures composed of components with dissimilar modes of toxic action, namely "independent action" (Bliss, 1939), whose applicability on community level effects was already shown (Backhaus et al., 2004). More information about the mixture predictions can be found in the Supporting Information. For statistics and figures, R version 3.0.2 together with the add-on packages "drc", "multcomp", "plotrix" and "vegan" was used (R Development Core Team, 2015).

RESULTS AND DISCUSSION

MICROBIAL LEAF DECOMPOSITION

As hypothesized, microbial leaf decomposition was significantly negatively affected by azoxystrobin (at 100 and 500 µg/L; Fig. 1A; group means or medians on the original scale as well as all *P*-values are provided in Table S4), carbendazim (at ≥245 µg/L; Fig. 1B), and cyprodinil (at ≥200 µg/L; Fig. 1C), while the observed effect sizes cannot be explained by the mass of fungicides potentially adsorbed to the leaf discs (Table S6). Since aquatic hyphomycete species – the main drivers of microbial decomposition (Gessner et al., 2007) – differ in their leaf decomposition efficiency (Duarte et al., 2006), fungicide-induced changes in fungal community composition may be the reason for this functional response. Accordingly, fungal communities exposed to these three substances separated well from the respective controls, with statistically significant differences in their composition (judged with PERMANOVA) being observed for azoxystrobin (at ≥20 µg/L) and carbendazim (at ≥35 µg/L). For cyprodinil, statistically non-significant but considerable shifts in the fungal community composition were found at concentrations ≥40 µg/L (Figs. S1 and S2), being corroborated by a significantly reduced number of leaf-associated fungal species (at ≥40 µg/L; Table S8).

In contrast, quinoxyfen and tebuconazole did not negatively affect microbial leaf decomposition (Figs. 1D, E). However, 1 µg tebuconazole/L unexpectedly resulted in a significantly higher decomposition compared to the control, which may also be considered an ecological adverse effect since it may increase competition between leaf-associated microorganisms and shredders and alter the timing regarding the availability of leaf material (Bärlocher, 1980; Greenwood et al., 2007). This effect may be triggered by the observed trend to an increase in fungal biomass at this concentration (Fig. 2E), which is potentially mediated indirectly via a reduced competitive pressure due to the depressed bacterial density (Fig. 2E). This negative effect on leaf-associated bacteria may be related to direct toxic effects of tebuconazole due to interactions with 14-α-demethylase, which is coded by the CYP51 gene, whose presence was demonstrated in several bacterial species (Režen et al., 2004). However, at 5 µg tebuconazole/L, where both the increase in fungal biomass and the reduction in bacterial density were statistically significant, microbial decomposition returned to the control level (Fig. 1E). This might be related either to the further reduction in the density of bacteria (Fig. 2E), which also contribute meaningfully to leaf decomposition (although much less than fungi; Hieber and

Gessner, 2002), or to effects on fungal groups other than aquatic hyphomycetes (e.g., Chytridiomycota), which were recently hypothesized to be involved in decomposition but were not determined during this study (Marano et al., 2011). In this context, molecular biological tools such as next-generation sequencing in combination with species- or group-specific quantitative real-time polymerase

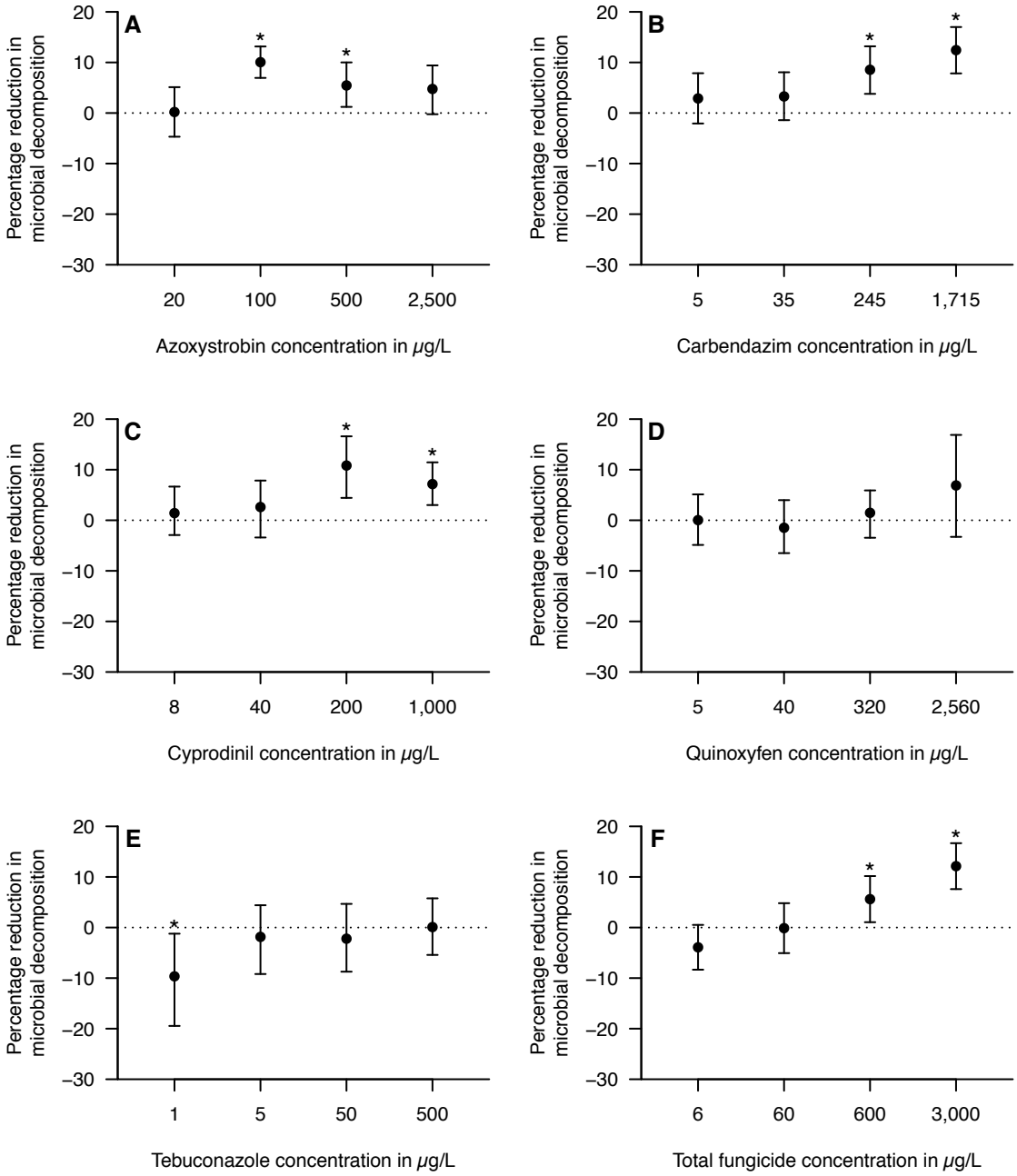

Fig. 1. Mean or median (with 95% CI) percentage reductions (compared to the respective control) in microbial decomposition of leaf material conditioned in the presence of different concentrations of A) azoxystrobin, B) carbendazim, C) cyprodinil, D) quinoxyfen, E) tebuconazole, and F) the fungicide mixture. Asterisks denote statistically significant differences compared to the respective control.

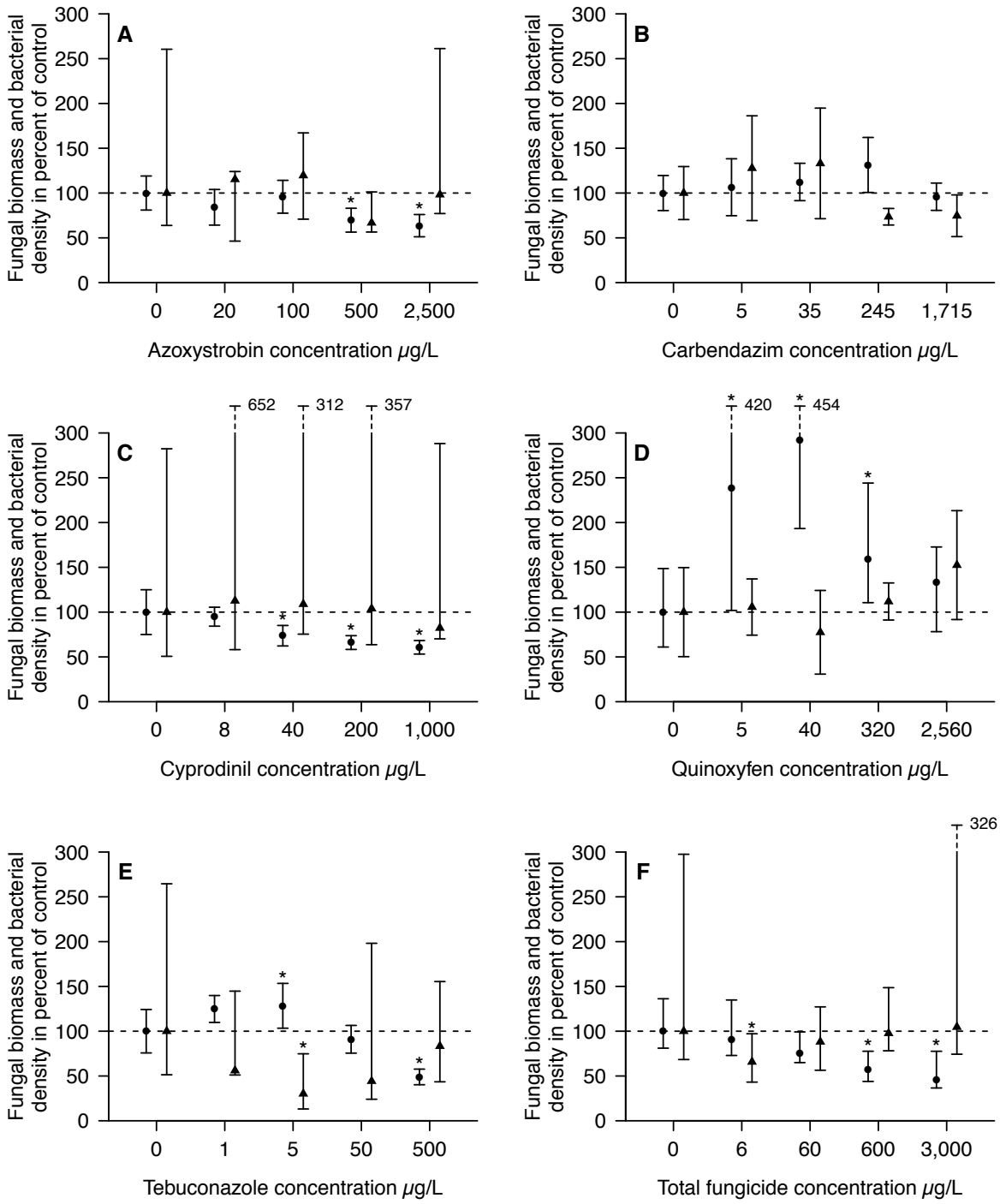

Fig. 2. Mean or median (with 95% CI) fungal biomass (circles) and bacterial density (triangles) relative to the respective control associated with leaf material conditioned in the presence of different concentrations of A) azoxystrobin, B) carbendazim, C) cyprodinil, D) quinoxyfen, E) tebuconazole, and F) the fungicide mixture. Asterisks denote statistically significant differences to the respective control.

chain reaction (Lindahl et al., 2013; Manerkar et al., 2008), may open the door for a more in-depth understanding of fungicide-induced implications in leaf associated microbial communities as well as species' functional contributions.

LEAF PALATABILITY

In line with our hypothesis, all tested fungicides affected leaf palatability negatively. This effect was displayed by a statistically significant preference of *Gammarus* for control over fungicide-exposed leaves at the highest concentrations of carbendazim, cyprodinil, quinoxyfen, and tebuconazole (Fig. 3B-E). Moreover, similar trends were observed at lower concentrations of these substances and at the highest concentration of azoxystrobin (Fig. 3A). However, although the biomass of leaf-associated fungi is presumed to be the most palatable component of leaf material for most shredders (as reviewed by Suberkropp, 1992), the food-choice behavior of *Gammarus* did not always correspond with this endpoint (Fig. 2A-E).

Instead, since *Gammarus* spp. are capable of discriminating between different fungal species (Arsuffi and Suberkropp, 1989; Gonçalves et al., 2014), changes in aquatic hyphomycete community composition were suggested a better predictor for fungicide-induced changes in leaf palatability than total fungal biomass (Bundschuh et al., 2011a). Indeed, fungicide exposure tended to reduce the sporulation of fungal species known to be preferred by gammarids, such as *Alatospora acuminata* INGOLD (Arsuffi and Suberkropp, 1989) and *Tricladium angulatum* INGOLD (Bärlocher and Kendrick, 1973; Table S9). At the same time, a trend to a higher contribution of *Tetracladium marchalianum* DE WILDEMAN, a species rejected by gammarids (Arsuffi and Suberkropp, 1989; Gonçalves et al., 2014), to total fungal sporulation was observed (Fig. S1), indicating a higher importance of this species for leaf palatability (Bundschuh et al., 2011a). In addition, cyprodinil exposure initiated the sporulation of *Heliscus lugdunensis* SACCARDO & THERRY, another hyphomycete species avoided by gammarids (Arsuffi and Suberkropp, 1989).

However, the absence of a microorganism-related explanation for the food-choice response induced by quinoxyfen (Fig. 2D and Supporting Information) and the good agreement between the effect sizes observed in the main and the adsorption experiments (Fig. 3) indicate that a repellent effect caused by the fungicides adsorbed to the leaf material is the more likely explanation for the observed feeding patterns. Nonetheless, the alterations in aquatic hyphomycete communities indicate the potential for bottom up effects on shredders' physiological status when consuming fungicide-exposed leaf material over the long term (Zubrod et al., 2011). These implications may be corroborated by toxic effects of co-ingested fungicides and other chemical stressors adsorbed to the leaf material (Bundschuh et al., 2013; Zubrod et al., 2014).

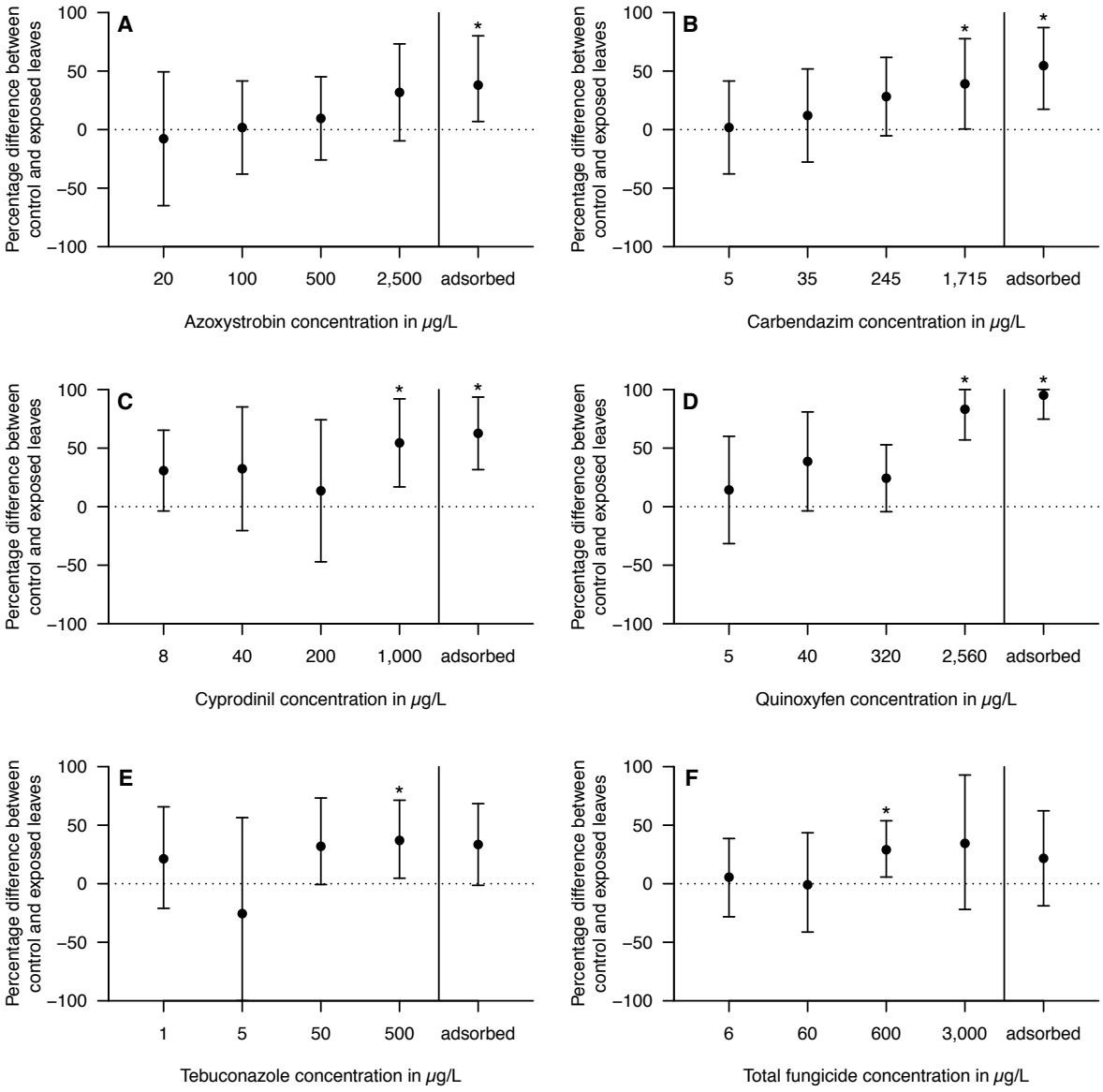

Fig. 3. Mean or median percentage difference (with 95% CI) in the feeding of *G. fossarum* on leaf discs conditioned in the presence of different concentrations of A) azoxystrobin, B) carbendazim, C) cyprodinil, D) quinoxyfen, E) tebuconazole, and F) the fungicide mixture compared to control discs. Positive effect sizes imply less feeding on the fungicide-exposed discs compared to the control and *vice versa*. Asterisks denote statistically significant differences compared to control leaf discs.

MIXTURE EFFECTS

Already at the lowest mixture concentration (i.e., 6 µg/L), we observed significant reductions in the number of fungal species (Tab. S8) and bacterial density (Fig. 2F). However, despite this and contrary to our expectation of mixture effects in accordance with or stronger than predictions by the reference model "independent action" (*cf.* Zubrod et al., 2014), a large fraction of the joint effects of the present study (Fig. 1F, 2F, and 3F) was lower than predicted (Table S10). A plausible explanation for this may be a positive nutritional effect of quinoxyfen for aquatic

hyphomycetes, which is indicated by a significantly increased leaf-associated fungal biomass when assessed singly (Fig. 2D). This may be related to the exceptional enzyme inventory of these fungi allowing them to mineralize organic xenobiotics (Krauss et al., 2011) such as quinoxyfen (Cabras et al., 2000) and utilize the stored energy (Fig. 2D). Additionally, leaf-associated fungi may have benefitted from the potentially reduced competitive pressure from bacteria as a result of tebuconazole exposure (Fig. 2D, E). As a consequence, aquatic hyphomycetes may have been capable of mitigating the negative effects of the remaining mixture components to some extent, for instance via energetic investments in detoxification (Sole et al., 2012). Such investments would result in less energy available for biomass accrual and could thus also explain the absence of the expected increase in fungal biomass (Fig. 2F; Table S10). However, this conclusion needs to be treated with caution since our experimental design involved the use of microbial inocula from different seasons, which was unavoidable due to logistic reasons and resulted in fungal communities differing among the controls of the six experiments (Table S11; see also Table S9 and Fig. S1). In consequence, communities potentially varying in their sensitivity to fungicide stress were used and thus tested at a point in time not necessarily representing their most sensitive state (as also hypothesized by Zubrod et al., 2015), which might affect the interpretation of the mixture results of the present study.

IMPLICATIONS FOR ERA

The European Union's ERA of fungicides is a tiered approach, whose first step involves a standard set of acute and chronic toxicity tests (EFSA, 2013). When comparing the ecotoxicological benchmarks from such standard tests (i.e., effective and lethal concentrations as well as no-observed effect concentrations – NOECs – extracted from the Pesticide Properties DataBase; FOOTPRINT, 2014) with the NOECs from the present study, not surprisingly aquatic hyphomycetes' sensitivity towards all tested fungicides was comparable to or even higher than that of the most sensitive standard test organism (Table 2). Note that it was often not even possible to determine a definitive NOEC due to statistically significant effects already at the lowest fungicide concentrations.

During first-tier ERA, safety factors of ten (for chronic experiments) or 100 (for acute experiments) are applied to the ecotoxicological benchmarks and the resulting regulatory acceptable concentrations (RACs) are finally compared to the predicted environmental concentration (EFSA, 2013). Indeed, RACs, determined during the

first tier, appear to provide a sufficient level of protection for the majority of structural and functional effects observed for individual fungicides in the present study (Table 2). The only obvious exception to this was tebuconazole, causing effects already at a concentration of 1 µg/L, which equals the RAC and is to the best of the authors' knowledge the lowest ever reported effective fungicide concentration with regard to a functional response in aquatic ecosystems. This further supports the high sensitivity of aquatic fungi towards demethylation-inhibiting fungicides (Artigas et al., 2012; Bundschuh et al., 2011a; Dijksterhuis et al., 2011; Dimitrov et al., 2014; Flores et al., 2014; Zubrod et al., 2011), a group accounting for approximately 20% of all synthetic fungicides approved in the European Union (see Supporting Information). Since comparably low concentrations were not tested for the remaining fungicides, similar effect patterns cannot be excluded.

Table 2. Toxicity endpoints (extracted from the Pesticide Properties DataBase; FOOTPRINT, 2014) from standard toxicity tests with the resulting regulatory acceptable concentrations (RAC; endpoints used for calculation are printed in bold) for the assessed substances. In addition, NOECs from this study are provided together with the most sensitive endpoint(s). All concentrations are given in µg/L.

Fungicide	Acute standard tests		Chronic standard tests				RAC	NOEC	End-point(s)[a]
	Daphnia	Fish	Algae	*Chironomus*	*Daphnia*	Fish			
Azoxystrobin	**230**	470	360	800	44	147	2.3	<20	FCC
Carbendazim	150	190	>7,700	13.3	**1.5**	3.2	0.15	5	FCC; FS; FSP
Cyprodinil	220	2,410	2,600	240	**8.8**	83	0.88	8	FB; FS
Quinoxyfen	**80**	270	27	128	28	14	0.8	<5	FB[b]
Tebuconazole	2,790	4,400	1,960	2,510	**10**	12	1	<1	MLD[b]

[a] FB fungal biomass; FCC fungal community composition; FS fungal species per sample; FSP fungal spore production; MLD microbial leaf decomposition

[b] Note that NOEC values of these fungicides are based on positive responses

Moreover, if a risk was indicated during the first tier, one option for a refined ERA is higher-tier effect assessment (EFSA, 2013). This procedure, which was used to assess the risk of another 20% of the synthetic fungicides (excluding demethylation-inhibiting fungicides) approved in the European Union, results in a median increase in RACs by a factor of approximately ten compared to the first tier (see Supporting Information). When applying this factor, all RACs of the fungicides tested during this study (with the exception of carbendazim) exceeded the corresponding NOECs (Table 2). Thus, our analyses indicate that the European Union's current ERA practices for individual fungicides may provide insufficient protection for aquatic

hyphomycetes for up to 40% of the approved substances (i.e., demethylation-inhibiting fungicides and substances assessed via higher-tier effect assessment), while it must be noted that the tested fungal communities were not necessarily in their most sensitive state for all fungicides (Table S11).

However, fungicides are barely detected as individual substances in the field (Bereswill et al., 2012; Reilly et al., 2012), and the above-described situation may be even worse for fungicide mixtures considering the significantly reduced number of fungal species already at the lowest tested mixture concentration of 6 µg/L (Table S8; compare with fungicide concentrations in surface water bodies of up to 80 µg/L; Bereswill et al., 2012). At this sum concentration, the concentrations of each individual component approximated the respective first-tier RAC (with the exception of carbendazim; Table 2). This questions the protection granted by these values and consequently also those derived by the less conservative higher-tier approaches for fungicide mixtures in general (Schäfer et al., 2012), particularly as fungicide cocktails in the field can contain an even higher number of substances than tested here (Bereswill et al., 2012; Reilly et al., 2012).

In future, the contamination of aquatic ecosystems with fungicides may locally even aggravate due to the expected intensification of fungicide application as a result of global climate change and invasive fungal pathogens (Hakala et al., 2011; Stokstad, 2004). Thus, it will be all the more important to have a fungicide ERA at hand that provides protective estimates of fungicide field concentrations (but see Knäbel et al., 2013) and their effects on aquatic key organisms. Since the present study's results suggest that the latter is not the case for aquatic hyphomycetes, their inclusion in ERA testing schemes seems mandatory to safeguard aquatic ecosystem functioning.

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SUPPORTING INFORMATION

APPLIED CONCENTRATIONS IN THE MIXTURE

Table S1. Concentrations of each fungicide applied in the different mixture treatments. All concentrations are given in µg/L.

Fungicide	Mixture concentration			
	6	60	600	3,000
Azoxystrobin	1	10	100	500
Carbendazim	1	10	100	500
Cyprodinil	1	10	100	500
Quinoxyfen	2	20	200	1,000
Tebuconazole	1	10	100	500

RESULTS OF FUNGICIDE ANALYSIS

Table S2. Nominal and measured (means with 95% confidence intervals (CI)) fungicide concentrations together with the respective limits of detection (LODs) and limits of quantification (LOQs).

			Single-substance experiments				Mixture experiment			
Fungicide	LOD (µg/L)	LOQ (µg/L)	Nominal (µg/L)	Measured at start (µg/L)	Measured after three days (µg/L)	% present after three days	Nominal (µg/L)	Measured at start (µg/L)	Measured after three days (µg/L)	% present after three days
Azoxystrobin	0.1	0.2	0.0	<LOD	<LOD	-	0.0	<LOD	<LOD	-
			20.0	19.8 (18.1 to 21.5)	8.1 (6.6 to 9.6)	40.8	1.0	0.9 (0.8 to 0.9)	0.4 (0.4 to 0.4)	45.9
			2,500.0	2,566.2 (1658.8 to 3473.7)	1,225.8 (847.5 to 1604.1)	47.8	500.0	460.8 (342.7 to 578.9)	220.5 (140.2 to 300.7)	47.8
Carbendazim	0.2	0.4	0.0	<LOD	<LOD	-	0.0	<LOD	<LOD	-
			5.0	4.6 (4.2 to 5.0)	3.4 (0.0 to 8.6)	74.2	1.0	0.8 (0.4 to 1.1)	0.7 (0.4 to 1.0)	96.1
			1,715.0	1,733.5 (1094.3 to 2372.6)	1,325.6 (963.5 to 1687.6)	76.5	500.0	463.9 (205.7 to 722.1)	356.6 (269.4 to 443.9)	76.9
Cyprodinil	0.2	0.6	0.0	<LOD	<LOD	-	0.0	<LOD	<LOD	-
			8.0	7.2 (5.4 to 9.0)	6.6 (6.1 to 7.0)	90.5	1.0	0.9 (0.7 to 1.0)	<LOD	<22.7
			1,000.0	1,137.1 (829.0 to 1445.2)	252.4 (191.3 to 313.5)	22.2	500.0	466.2 (252.0 to 680.3)	23.0 (19.8 to 26.3)	4.9
Quinoxyfen	0.1	0.3	0.0	<LOD	<LOD	-	0.0	<LOD	<LOD	-
			5.0	5.4 (5.2 to 5.5)	0.8 (0.6 to 0.9)	14.0	2.0	2.3 (1.8 to 2.8)	<LOQ	<13.1
			2,560.0	2,982.3 (2167.0 to 3797.6)	31.6 (0.0 to 68.4)	1.1	1000.0	906.5 (801.9 to 1011.1)	5.3 (4.3 to 6.3)	0.6
Tebuconazole	0.2	0.60	0.0	<LOD	<LOD	-	0.0	<LOD	<LOD	-
			1.0	1.1 (0.8 to 1.3)	<LOQ	<56.6	1.0	1.1 (0.6 to 1.5)	<LOQ	<57.1
			500.0	467.1 (245.5 to 688.7)	201.9 (120.8 to 283.0)	43.2	500.0	502.2 (361.2 to 643.2)	190.9 (166.9 to 214.8)	38.0

COMPOSITION OF TEST MEDIA

Table S3. Composition of the conditioning medium and SAM-5S (amphipod medium). The pH of conditioning medium was adjusted to 7.0.

Salt	Concentration in conditioning medium according to Dang et al. (2005; in mg/L)	Concentration in SAM-5S according to Borgmann (1996; in mg/L)
$CaCl_2$ x $2H_2O$	100	147
$MgSO_4$ x $7H_2O$	10	61.5
Morpholino propane sulfonic acid	500	-
KNO_3	100	-
K_2HPO_4	5.5	-
$NaHCO_3$	-	85.5
KCl	-	3.8
NaBr	-	1.03

ESTIMATES AND *P*-VALUES FROM COMPARISONS PRESENTED IN THE MANUSCRIPT

Table S4. Group means or medians (if applicable; with 95% CI) on the original scale as well as *P*-values from statistical comparisons of fungicide treatments with the respective control together with the statistical test used for their calculation. All *P*-values <0.05 are printed in bold.

Fungicide	Concentration (µg/L)	Endpoint	Mean or median	95% CI	Statistical test	*P*-value
Azoxystrobin	0 (for 20)	Microbial leaf decomposition in mg/day	0.20	0.19-0.22	Wilcoxon signed-rank	0.953
	20		0.20	0.19-0.22		
	0 (for 100)		0.23	0.21-0.24		**<0.001**
	100		0.20	0.19-0.22		
	0 (for 500)		0.19	0.18-0.23		**0.011**
	500		0.19	0.18-0.21		
	0 (for 2,500)		0.24	0.22-0.25		0.055
	2,500		0.22	0.20-0.23		
	0 (for 20)	Gammarids' leaf consumption in mg/mg/day	0.24	0.15-0.33	Paired Student's *t*	0.784
	20		0.26	0.18-0.33		
	0 (for 100)		0.32	0.23-0.41		0.930
	100		0.32	0.23-0.40		
	0 (for 500)		0.32	0.25-0.39		0.590
	500		0.29	0.22 0.35		
	0 (for 2,500)		0.33	0.24-0.42		0.129
	2,500		0.22	0.13-0.32		
	0 (for add-on experiment)		0.27	0.14-0.42	Wilcoxon signed-rank	**0.018**
	Add-on experiment		0.14	0.12-0.19		
	0	Fungal biomass in mg ergosterol/g	147.9	124.8-170.9	Dunnett's *t*	
	20		123.5	88.5-158.5		0.325
	100		143.1	115.7-170.4		0.982
	500		104.0	84.2-123.9		**0.021**
	2,500		94.9	76.4-113.5		**0.007**
	0	Fungal community composition	-	-	PERMANOVA with Bonferroni adjustment	
	20		-	-		**0.032**
	100		-	-		**0.016**
	500		-	-		**0.004**
	2,500		-	-		**0.004**
	0	Bacterial density in 10^8 cells/mg	3.1	2.0-8.1	Wilcoxon rank-sum with Bonferroni adjustment	
	20		3.6	1.4-3.8		1.000
	100		3.7	2.2-5.2		1.000
	500		2.1	1.8-3.1		0.638
	2,500		3.0	2.4-8.1		1.000

Table S4 continued.

Fungicide	Concentration (µg/L)	Endpoint	Mean or median	95% CI	Statistical test	*P*-value
Carbendazim	0 (for 5)	Microbial leaf decomposition in mg/day	0.30	0.26-0.33	Wilcoxon signed-rank	0.243
	5		0.31	0.27-0.33		
	0 (for 35)		0.28	0.24-0.32		0.166
	35		0.28	0.22-0.30		
	0 (for 245)		0.30	0.27-0.35		**<0.001**
	245		0.29	0.23-0.31		
	0 (for 1,715)		0.32	0.28-0.34		**<0.001**
	1,715		0.30	0.24-0.31		
	0 (for 5)	Gammarids' leaf consumption in mg/mg/day	0.24	0.17-0.32	Paired Student's *t*	0.927
	5		0.24	0.17-0.31		
	0 (for 35)		0.23	0.18-0.29		0.545
	35		0.21	0.14-0.27		
	0 (for 245)		0.30	0.21-0.39		0.097
	245		0.21	0.16-0.26		
	0 (for 1,715)		0.24	0.16-0.31		**0.047**
	1,715		0.14	0.10-0.19		
	0 (for add-on experiment)		0.19	0.15-0.33	Wilcoxon signed-rank	**0.002**
	Add-on experiment		0.09	0.06-0.17		
	0	Fungal biomass in mg ergosterol/g	132.2	106.3-158.0		
	5		140.7	98.7-182.7		0.973
	35		148.5	120.9-176.1	Dunnett's *t*	0.796
	245		173.6	133.0-214.2		0.109
	1,715		126.7	106.6-146.9		0.995
	0	Fungal community composition	-	-	PERMANOVA with Bonferroni adjustment	
	5		-	-		1.000
	35		-	-		**0.004**
	245		-	-		**0.008**
	1,715		-	-		**0.004**
	0	Bacterial density in 10^8 cells/mg	3.6	2.5-4.6		
	5		4.6	2.5-6.7		0.611
	35		4.8	2.6-7.0	Dunnett's *t*	0.463
	245		2.6	2.3-3.0		0.649
	1,715		2.7	1.8-3.5		0.679
Cyprodinil	0 (for 8)	Microbial leaf decomposition in mg/day	0.18	0.17-0.19	Wilcoxon signed-rank	0.521
	8		0.18	0.16-0.19		
	0 (for 40)		0.19	0.17-0.21		0.369
	40		0.18	0.17-0.20		
	0 (for 200)		0.19	0.15-0.20		**0.001**
	200		0.17	0.13-0.18		
	0 (for 1,000)		0.20	0.19-0.23		**<0.001**
	1,000		0.19	0.17-0.20		

Table S4 continued.

Fungicide	Concentration (µg/L)	Endpoint	Mean or median	95% CI	Statistical test	*P*-value
Cyprodinil	0 (for 8)	Gammarids' leaf consumption in mg/mg/day	0.34	0.27-0.42	Paired Student's *t*	0.079
	8		0.24	0.16-0.31		
	0 (for 40)		0.29	0.19-0.39		0.223
	40		0.20	0.09-0.31		
	0 (for 200)		0.38	0.22-0.54		0.655
	200		0.33	0.19-0.47		
	0 (for 1,000)		0.38	0.27-0.49		**0.005**
	1,000		0.17	0.09-0.26		
	0 (for add-on experiment)		0.41	0.31-0.52		**<0.001**
	Add-on experiment		0.15	0.11-0.20		
	0	Fungal biomass in mg ergosterol/g	229.2	171.9-286.4	Dunnett's *t*	
	8		217.5	193.3-241.7		0.865
	40		168.8	142.6-195.0		**0.003**
	200		151.3	133.7-168.9		**<0.001**
	1,000		139.2	121.9-156.5		**<0.001**
	0	Fungal community composition	-	-	PERMANOVA with Bonferroni adjustment	
	8		-	-		1.000
	40		-	-		0.112
	200		-	-		0.120
	1,000		-	-		0.084
	0	Bacterial density in 10^8 cells/mg	2.0	1.0-5.7	Wilcoxon rank-sum with Bonferroni adjustment	
	8		2.3	1.2-13.2		1.000
	40		2.2	1.5-6.3		1.000
	200		2.1	1.3-7.2		1.000
	1,000		1.7	1.4-5.9		1.000
Quinoxyfen	0 (for 5)	Microbial leaf decomposition in mg/day	0.34	0.31-0.37	Wilcoxon signed-rank	0.988
	5		0.35	0.31-0.38		
	0 (for 40)		0.35	0.31-0.37		0.539
	40		0.36	0.31-0.40		
	0 (for 320)		0.35	0.33-0.37		0.508
	320		0.35	0.33-0.36		
	0 (for 2,560)		0.34	0.31-0.39		0.182
	2,560		0.31	0.30-0.34		
	0 (for 5)	Gammarids' leaf consumption in mg/mg/day	0.29	0.22-0.36	Paired Student's *t*	0.533
	5		0.25	0.14-0.35		
	0 (for 40)		0.30	0.22-0.39		0.072
	40		0.19	0.09-0.28		
	0 (for 320)		0.44	0.35-0.52		0.094
	320		0.33	0.25-0.41		
	0 (for 2,560)		0.54	0.40-0.69		**<0.001**
	2,560		0.09	0.05-0.14		
	0 (0 for add-on experiment)		0.65	0.54-0.76		**<0.001**
	Add-on experiment		0.03	0.00-0.07		

Table S4 continued.

Fungicide	Concentration (µg/L)	Endpoint	Mean or median	95% CI	Statistical test	*P*-value
Quinoxyfen	0	Fungal biomass in mg ergosterol/g	86.4	52.8-128.5	Wilcoxon rank-sum with Bonferroni adjustment	
	5		206.4	88.1-362.9		**0.028**
	40		252.7	167.1-392.8		**0.002**
	320		137.3	95.6-211.0		**0.016**
	2,560		115.0	67.6-149.3		1.000
	0	Fungal community composition	-	-	PERMANOVA with Bonferroni adjustment	
	5		-	-		1.000
	40		-	-		1.000
	320		-	-		1.000
	2,560		-	-		1.000
	0	Bacterial density in 10^8 cells/mg	4.2	2.1-6.3	Dunnett's *t*	
	5		4.4	3.1-5.7		0.998
	40		3.3	1.3-5.2		0.791
	320		4.7	3.8-5.6		0.972
	2,560		6.4	3.8-8.9		0.148
Tebuconazole	0 (for 1)	Microbial leaf decomposition in mg/day	0.19	0.17-0.21	Wilcoxon signed-rank	**0.028**
	1		0.19	0.18-0.23		
	0 (for 5)		0.22	0.19-0.24		0.596
	5		0.21	0.20-0.22		
	0 (for 50)		0.21	0.19-0.22		0.515
	50		0.22	0.20-0.23		
	0 (for 500)		0.23	0.20-0.24		0.984
	500		0.23	0.20-0.24		
	0 (for 1)	Gammarids' leaf consumption in mg/mg/day	0.25	0.16-0.31	Wilcoxon signed-rank	0.300
	1		0.23	0.11-0.26		
	0 (for 5)		0.21	0.09-0.28		0.427
	5		0.23	0.10-0.36		
	0 (for 50)		0.29	0.16-0.39		0.054
	50		0.17	0.10-0.30		
	0 (for 500)		0.35	0.22-0.47		**0.027**
	500		0.20	0.11-0.36		
	0 (0 for add-on experiment)		0.45	0.35-0.55	Paired Student's *t*	0.059
	Add-on experiment		0.30	0.22-0.37		
	0	Fungal biomass in mg ergosterol/g	138.8	105.1-172.4	Dunnett's *t*	
	1		173.2	152.4-194.0		0.073
	5		178.1	143.4-212.8		**0.043**
	50		126.3	104.8-147.7		0.797
	500		68.0	55.9-80.1		**<0.001**

Table S4 continued.

Fungicide	Concentration (µg/L)	Endpoint	Mean or median	95% CI	Statistical test	*P*-value
Tebuconazole	0		-	-		
	1	Fungal community composition	-	-	PERMANOVA with Bonferroni adjustment	1.000
	5		-	-		1.000
	50		-	-		1.000
	500		-	-		**0.004**
	0	Bacterial density in 10^8 cells/mg	5.7	2.9-15.0	Wilcoxon rank-sum with Bonferroni adjustment	
	1		3.2	2.9-8.2		1.000
	5		1.7	0.7-4.2		**0.016**
	50		2.5	1.4-11.2		0.152
	500		4.7	2.5-8.8		1.000
Fungicide mixture	0 (for 6)	Microbial leaf decomposition in mg/day	0.20	0.18-0.21	Paired Student's *t*	0.082
	6		0.21	0.19-0.22		
	0 (for 60)		0.21	0.20-0.23		0.959
	60		0.21	0.20-0.23		
	0 (for 600)		0.20	0.18-0.22		**0.017**
	600		0.19	0.17-0.20		
	0 (for 3,000)		0.26	0.24-0.29		**<0.001**
	3,000		0.23	0.21-0.25		
	0 (for 6)	Gammarids' leaf consumption in mg/mg/day	0.17	0.14-0.24	Wilcoxon signed-rank	0.814
	6		0.21	0.14-0.26		
	0 (for 60)		0.21	0.12-0.32		0.949
	60		0.23	0.15-0.31		
	0 (for 600)		0.31	0.17-0.38		**0.014**
	600		0.17	0.06-0.29		
	0 (for 3,000)		0.17	0.11-0.24		0.227
	3,000		0.12	0.00-0.25		
	0 (for add-on experiment)		0.39	0.29-0.49	Paired Student's *t*	0.287
	Add-on experiment		0.31	0.21-0.40		
	0	Fungal biomass in mg ergosterol/g	264.6	217.3-311.9	Dunnett's *t*	
	6		251.9	198.8-305.0		0.953
	60		218.3	184.0-252.6		0.172
	600		159.4	128.4-190.5		**<0.001**
	3,000		128.5	96.4-160.7		**<0.001**
	0	Fungal community composition	-	-	PERMANOVA with Bonferroni adjustment	
	6		-	-		1.000
	60		-	-		0.220
	600		-	-		**0.012**
	3,000		-	-		**0.004**
	0	Bacterial density in 10^8 cells/mg	4.5	3.1-13.5	Wilcoxon rank-sum with Bonferroni adjustment	
	6		3.0	2.0-4.4		**0.044**
	60		4.0	2.6-5.8		1.000
	600		4.4	3.5-6.7		1.000
	3,000		4.7	3.4-14.8		1.000

MASS OF FUNGICIDES POTENTIALLY ADSORBED TO LEAF DISCS

All fungicides were applied up to very high concentrations (up to 3,000 µg/L), being partly of low water solubility and highly affine to organic material (Table S5). One may thus assume that the reported effects on microbial leaf decomposition may actually be artifacts from adsorption of the fungicides to the leaf discs. The following calculations show that this is not the case:

One aquarium contained 68 leaf discs with a leaching-corrected mean dry weight of 8.8 mg/disc (8.7 to 8.9; 95% CI) prior to conditioning, which was estimated from the pre-weighed discs for the assessment of functional endpoints. Assuming a dry weight of 1.8 g for the inoculum in the aquaria as reported by Zubrod et al. (2015), the 68 leaf discs account for only 25% of the leaf material present. For a worst-case estimation, adsorption (*A*) of the single substances was assumed to be *A = 100% - minimum (% present after three days; from Table S1)*. Given four medium renewals per experiment, the mass (*M*) of the fungicide(s) that could adsorb to one leaf disc is thus given by *M (µg) = nominal concentration (µg/L) x A x 4 L / 68* (25% and 4 medium renewals cancel out). If corrected for this potential but (considering the calculated size) unlikely bias, still statistically significant effects can be observed in all cases that were judged as significant before (Table S6).

Table S5. Water solubility and affinity to organic material (i.e., K_{OC}-values) of the tested fungicides according to the Pesticide Action Network Pesticide Database (2014).

Fungicide	Water solubility (mg/L)	K_{OC}
Azoxystrobin	6.00	581
Carbendazim	8.00	223
Cyprodinil	16.00	1,470
Quinoxyfen	0.05	22,929
Tebuconazole	32.00	1,000

Table S6. Mass of fungicides potentially adsorbed to leaf discs as well as original and bias-corrected effect sizes. Corrected *P*-values stem from paired *t*-tests or Wilcoxon signed-rank tests for comparisons of bias-corrected microbial decomposition with the respective control. All *P*-values <0.05 are printed in bold.

Experiment	Concentration (µg/L)	Maximum adsorption (%)	Adsorbed fungicide mass (µg)	Original effect size (mg)	Corrected effect size (mg)	Original inhibition (%)	Corrected inhibition (%)	Corrected *P*-value
Azoxystrobin	20	59.2	0.70	0.01	0.01	0.20	0.18	0.961
	100		3.48	0.31	0.31	10.05	9.94	**<0.001**
	500		17.41	0.15	0.13	5.45	4.81	**0.027**
	2,500		87.06	0.14	0.06	4.75	1.89	0.370
Carbendazim	5	25.8	0.08	0.11	0.11	2.88	2.88	0.243
	35		0.53	0.12	0.12	3.28	3.27	0.169
	245		3.72	0.34	0.34	8.55	8.46	**0.001**
	1,715		26.03	0.51	0.49	12.44	11.81	**<0.001**
Cyprodinil	8	95.1	0.45	0.03	0.03	1.42	1.40	0.528
	40		2.24	0.07	0.06	2.62	2.53	0.385
	200		11.19	0.25	0.24	10.82	10.34	**0.001**
	1,000		55.94	0.19	0.14	7.15	5.08	**0.016**
Quinoxyfen	5	99.4	0.29	0.00	0.00	0.03	0.02	0.992
	40		2.34	-0.07	-0.07	-1.48	-1.53	0.512
	320		18.71	0.07	0.05	1.48	1.07	0.615
	2,560		149.68	0.30	0.15	6.89	3.48	0.410
Tebuconazole	1	62.0	0.04	-0.24	-0.24	-9.65	-9.65	**0.028**
	5		0.18	-0.05	-0.05	-1.85	-1.86	0.589
	50		1.82	-0.06	-0.06	-2.21	-2.27	0.508
	500		18.24	0.00	-0.02	0.09	-0.51	0.828
Fungicide mixture	6	See above	0.26	-0.10	-0.10	-3.91	-3.92	0.081
	60		2.59	0.00	-0.01	-0.13	-0.22	0.929
	600		25.94	0.15	0.12	5.61	4.62	**0.047**
	3,000		129.68	0.41	0.28	12.13	8.33	**<0.001**

FURTHER INFORMATION ABOUT THE AQUATIC HYPHOMYCETE COMMUNITIES

In total, the spores of 28 aquatic hyphomycete species were identified during the present study (Table S7). Only six of these species contributed on average 91% to total sporulation in the controls (average contributions: *Clavariopsis aquatica* (4%), *Clavatospora longibrachiata* (24%), *Heliscella stellata* (11%), *Sigmoidea aurantiaca* (7%), *Tetracladium marchalianum* (37%), *Tricladium angulatum* (8%); Fig. S1).

Azoxystrobin exposure caused a significantly reduced number of fungal species per sample (≥100 µg/L) and spore production (≥500 µg/L; Table S8) resulting in almost exclusive sporulation by *T. marchalianum* already at the lowest tested concentration (Fig. S1A). Carbendazim triggered significant reductions in the two sporulation-related endpoints at concentrations ≥35 µg/L, while it completely inhibited fungal sporulation at 1,715 µg/L (Table S8). Again, *T. marchalianum*'s contribution to total sporulation strongly increased due to fungicide exposure (Fig. S1B). Cyprodinil significantly reduced the number of sporulating species at 40 µg/L, while a strong tendency was also observable at higher concentrations (Table S8). At lower concentrations again the contribution of *T. marchalianum* to spore production was increased compared to the control (Fig. S1C). Interestingly, starting at 40 µg/L, the spores of *Heliscus lugdunensis* could be identified, becoming the dominating species at higher concentrations, although not detected in the control or the 5-µg/L treatment. Quinoxyfen did not trigger any statistically significant changes in aquatic hyphomycete community structure. Tebuconazole significantly reduced the number of sporulating species at concentrations ≥50 µg/L (Table S8). For the fungicide mixture, a significant reduction in the number of fungal species identified per sample was observed at concentrations ≥6 µg/L (at 60 µg/L only as tendency; Table S8) and spore production was reduced at ≥600 µg/L (Table S8). The fungicide mixture tended to increase the contribution of *T. marchalianum* to total spore production and at the highest tested concentration only *C. longibrachiata* and *T. marchalianum* were able to sporulate (Fig. S1F). Moreover, with few exceptions, results of similarity percentage analysis (SIMPER; on square-root transformed data; Table S8) showed that the six dominant species also accounted for most of the dissimilarities between controls and fungicide treatments (Fig. S2).

Table S7. List of all aquatic hyphomycete species identified during the present study. Species in brackets were identified in only one sample of the experiment.

Species	Experiment					
	Azoxystrobin	Carbendazim	Cyprodinil	Quinoxyfen	Tebuconazole	Fungicide mixture
Alatospora acuminata Ingold	X	X	(X)	X	X	X
Alatospora constricta Dyko		X				X
Anguillospora crassa Ingold		(X)		(X)		
Anguillospora longissima (Saccardo & P. Sydow) Ingold	X	X		X	X	
Articulospora tetracladia Ingold	X				X	
Clavariopsis aquatica De Wildeman	X	X	X	X	X	X
Clavatospora longibrachiata (Ingold) Sv. Nilsson ex Marvanová & Sv. Nilsson	X	X	X	X	X	X
Flagellospora curvula Ingold	(X)	X	X	X	X	X
Geniculospora inflata (Ingold) Sv. Nilsson ex Marvanová & Sv. Nilsson	X				X	X
Heliscella stellata (Ingold & V.J. Cox) Marvanová	X	X	X	X	X	X
Heliscus lugdunensis Saccardo & Therry		X	X			(X)
Lemonniera aquatica De Wildeman		X				(X)
Lemonniera terrestris Tubaki	X				(X)	
Lunulospora curvula Ingold						X
Microstella pluvioriens K. Ando & Tubaki			X	(X)		X
Mycocentrospora clavata S.H. Iqbal	X				(X)	
Naiadella fluitans Marvanová & Bandoni	(X)					
Pseudoanguillospora stricta S.H. Iqbal	(X)					(X)
Sigmoidea aurantiaca Descals	X	X		X	X	X
Tetracladium marchalianum De Wildeman	X	X	X	X	X	X
Tetracladium setigerum (Grove) Ingold		(X)		(X)		
Tricladium angulatum Ingold	X	X	X	X	X	X
Tricladium gracile Ingold					(X)	
Tricladium patulum Marvanová & Marvan	X				X	
Tricladium terrestre D. Park	X				X	
Tripospermum myrti (Lind) S. Hughes	X				(X)	
Triscelophorus acuminatus Nawawi	(X)					
Triscelophorus monosporus Ingold	X	X			X	
Total number (species detected only once)	20 (4)	15 (2)	9 (1)	12 (3)	18 (4)	15 (3)

Table S8. Mean or median number of fungal species and spores (with 95% CIs). Reported *P*-values originate from Dunnett's tests or Bonferroni-adjusted Wilcoxon-rank sum tests for comparisons with the respective control. For SIMPER, the five species contributing most to dissimilarities between fungicide treatments and the respective control are reported. All *P*-values <0.05 are printed in bold.

Experiment	Concentration (µg/L)	Number of species per sample	*P*-value	Number of spores per mg leaf	*P*-value	SIMPER results with percentage contribution
Azoxystrobin	0	9.0 (7.4 to 10.6)		48.9 (9.9 to 116.8)		
	20	7.6 (6.0 to 9.1)	0.301	29.9 (11.3 to 262.1)	1.000	*T. marchalianum* (27.0); *H. stellata* (21.0); *S. aurantiaca* (14.1); *C. longibrachiata* (8.6); *T. angulatum* (8.2)
	100	6.0 (4.5 to 7.5)	**0.006**	54.7 (12.6 to 161.9)	1.000	*T. marchalianum* (24.3); *H. stellata* (23.7); *A. acuminata* (13.4); *S. aurantiaca* (13.1); *C. longibrachiata* (7.4)
	500	2.1 (0.5 to 3.8)	**<0.001**	4.9 (0.0 to 26.3)	**0.020**	*T. marchalianum* (26.3); *H. stellata* (23.8); *S. aurantiaca* (13.7); *C. longibrachiata* (11.4); *T. angulatum* (8.8)
	2,500	1.3 (0.3 to 2.3)	**<0.001**	0.2 (0.0 to 0.4)	**0.008**	*T. marchalianum* (31.6); *H. stellata* (21.2); *S. aurantiaca* (12.6); *C. longibrachiata* (10.8); *T. angulatum* (9.0)
Carbendazim	0	7.3 (6.1 to 8.4)		82.0 (30.4 to 550.4)		
	5	7.1 (6.3 to 8.0)	0.998	61.0 (19.3 to 153.6)	1.000	*C. longibrachiata* (28.0); *T. marchalianum* (14.4); *H. stellata* (13.9); *F. curvula* (10.9); *T. angulatum* (10.0)
	35	2.9 (1.1 to 4.6)	**<0.001**	3.5 (0.0 to 23.2)	**0.002**	*C. longibrachiata* (41.2); *H. stellata* (18.7); *T. marchalianum* (13.7); *T. angulatum* (9.4); *C. aquatica* (7.9)
	245	1.3 (0.6 to 2.0)	**<0.001**	0.3 (0.0 to 0.6)	**0.009**	*C. longibrachiata* (40.0); *H. stellata* (18.1); *T. marchalianum* (14.1); *T. angulatum* (11.6); *C. aquatica* (7.6)
	1,715	0.0 (0.0 to 0.0)	**<0.001**	0.0 (0.0 to 0.0)	**0.004**	*C. longibrachiata* (39.9); *H. stellata* (17.7); *T. marchalianum* (15.3); *T. angulatum* (11.4); *C. aquatica* (7.4) (note that these results represent a comparison between the control and seven empty samples)
Cyprodinil	0	2.6 (0.3 to 4.8)		0.3 (0.0 to 46.2)		
	8	1.3 (0.0 to 2.8)	0.321	0.1 (0.0 to 4.2)	1.000	*T. marchalianum* (34.1); *C. longibrachiata* (23.1); *H. stellata* (12.0); *C. aquatica* (12.0); *T. angulatum* (7.5)
	40	0.4 (0.0 to 1.2)	**0.038**	0.0 (0.0 to 0.1)	0.113	*T. marchalianum* (27.6); *C. longibrachiata* (25.4); *H. stellata* (18.0); *T. angulatum* (9.6); *C. aquatica* (7.0)
	200	0.6 (0.0 to 1.6)	0.057	0.0 (0.0 to 0.9)	0.505	*C. longibrachiata* (24.8); *T. marchalianum* (23.1); *H. lugdunensis* (14.9); *H. stellata* (14.0); *T. angulatum* (8.4)
	1,000	0.6 (0.0 to 1.3)	0.058	0.0 (0.0 to 0.7)	0.357	*C. longibrachiata* (22.9); *T. marchalianum* (22.9); *H. stellata* (14.1); *H. lugdunensis* (12.9); *T. angulatum* (8.2)
Quinoxyfen	0	5.3 (4.3 to 6.3)		3.1 (0.3 to 10.9)		
	5	5.6 (4.7 to 6.5)	0.991	5.9 (1.3 to 25.6)	1.000	*T. marchalianum* (28.7); *C. longibrachiata* (16.8); *A. acuminata* (16.2); *T. angulatum* (11.9); *H. stellata* (9.1)
	40	4.4 (2.8 to 6.1)	0.702	3.5 (0.4 to 12.7)	1.000	*C. longibrachiata* (26.6); *T. marchalianum* (22.5); *T. angulatum* (13.8); *A. acuminata* (9.8); *H. stellata* (9.2)
	320	5.6 (4.7 to 6.5)	0.991	1.1 (0.7 to 2.6)	0.152	*T. marchalianum* (23.9); *C. longibrachiata* (17.4); *C. aquatica* (12.3) ; *A. acuminata* (11.0); *H. stellata* (10.9)
	2,560	5.0 (2.7 to 7.3)	0.991	3.3 (0.0 to 6.7)	1.000	*T. marchalianum* (23.4); *C. longibrachiata* (18.8); *T. angulatum* (14.7); *A. acuminata* (12.0); *H. stellata* (10.1)
Tebuconazole	0	9.7 (8.1 to 11.3)		54.3 (7.7 to 148.2)		
	1	9.0 (8.1 to 9.9)	0.771	26. 5 (6.5 to 80.4)	0.834	*T. marchalianum* (29.9); *S. aurantiaca* (14.9); *T. angulatum* (12.7); *C. aquatica* (9.2); *C. longibrachiata* (6.5)
	5	8.6 (7.1 to 10.1)	0.408	63.6 (5.8 to 114.0)	1.000	*T. marchalianum* (32.9); *T. angulatum* (15.1); *S. aurantiaca* (14.7); *C. aquatica* (9.1); *C. longibrachiata* (6.0)
	50	7.4 (5.8 to 9.1)	**0.023**	43.5 (4.3 to 189.6)	1.000	*T. marchalianum* (37.0); *S. aurantiaca* (14.1); *T. angulatum* (11.2); *C. aquatica* (9.1); *H. stellata* (6.1)
	500	4.7 (3.8 to 5.6)	**<0.001**	14.2 (11.7 to 79.0)	0.660	*T. marchalianum* (30.2); *T. angulatum* (13.9); *C. aquatica* (12.8); *S. aurantiaca* (12.5); *C. longibrachiata* (8.4)
Fungicide	0	6.9 (5.4 to 8.3)		10.7 (3.2 to 34.8)		
mixture	6	4.9 (2.9 to 6.8)	**0.040**	6.2 (2.1 to 14.8)	0.660	*T. marchalianum* (21.0); *A. constricta* (16.1); *C. longibrachiata* (15.1); *G. inflata* (11.7); *S. aurantiaca* (10.7)
	60	5.0 (3.7 to 6.3)	0.061	12.4 (1.8 to 20.6)	1.000	*T. marchalianum* (24.9); *A. constricta* (16.2); *C. longibrachiata* (14.6); *S. aurantiaca* (10.9); *G. inflata* (9.6)
	600	1.3 (0.6 to 2.0)	**<0.001**	0.6 (0.0 to 7.3)	**0.013**	*T. marchalianum* (23.4); *A. constricta* (19.1); *C. longibrachiata* (17.3); *S. aurantiaca* (11.7); *G. inflata* (7.6)
	3,000	0.3 (0.0 to 0.7)	**<0.001**	0.0 (0.0 to 0.1)	**0.007**	*T. marchalianum* (26.7); *A. constricta* (19.2); *C. longibrachiata* (16.1); *S. aurantiaca* (11.3); *G. inflata* (7.1)

Table S9. Median number of aquatic hyphomycete spores per sample (with 95% CIs) from species accounting for ≥1% of all spores in at least one treatment of the respective experiment. Reported *P*-values stem from Bonferroni-adjusted Wilcoxon-rank sum tests for comparisons with the respective control. All *P*-values <0.05 are printed in bold.

Experiment	Concentration (µg/L)	*A. acuminata*		*A. constricta*		*A. longissima*		*C. aquatica*		*C. longibrachiata*		*F. curvula*		*H. stellata*	
		Spores	*P*-value	Spores	*P*-value	Spores	*P*-value	Spores	*P*-value	Spores	*P*-value	Spores	*P*-value	Spores	*P*-value
Azoxystrobin	0	1 (0 to 88)								27 (6 to 226)				101 (37 to 1360)	
	20	4 (2 to 18)	1.000							9 (1 to 20)	**0.044**			12 (1 to 122)	**0.042**
	100	14 (1 to 437)	0.549							7 (2 to 23)	**0.042**			0 (0 to 1)	**0.006**
	500	0 (0 to 22)	0.660							0 (0 to 4)	**0.007**			0 (0 to 0)	**0.004**
	2,500	0 (0 to 0)	**0.014**							0 (0 to 0)	**0.004**			0 (0 to 0)	**0.004**
Carbendazim	0	0 (0 to 32)						18 (0 to 288)		463 (153 to 7281)		0 (0 to 2)		128 (4 to 882)	
	5	6 (0 to 45)	0.854					20 (0 to 270)	1.000	751 (90 to 2682)	1.000	9 (0 to 304)	0.094	113 (20 to 567)	1.000
	35	0 (0 to 11)	1.000					0 (0 to 0)	**0.015**	1 (0 to 18)	**0.008**	0 (0 to 1)	1.000	0 (0 to 5)	**0.009**
	245	0 (0 to 5)	1.000					0 (0 to 0)	**0.015**	0 (0 to 3)	**0.008**	0 (0 to 0)	1.000	0 (0 to 0)	**0.004**
	1,715	0 (0 to 0)	0.302					0 (0 to 0)	**0.015**	0 (0 to 0)	**0.004**	0 (0 to 0)	1.000	0 (0 to 0)	**0.004**
Cyprodinil	0							0 (0 to 10)		0 (0 to 714)		0 (0 to 17)		0 (0 to 22)	
	8							0 (0 to 37)	1.000	0 (0 to 2)	1.000	0 (0 to 1)	1.000	0 (0 to 0)	0.302
	40							0 (0 to 0)	0.302	0 (0 to 0)	0.692	0 (0 to 0)	0.692	0 (0 to 1)	0.802
	200							0 (0 to 0)	0.302	0 (0 to 1)	1.000	0 (0 to 0)	0.692	0 (0 to 0)	0.302
	1,000							0 (0 to 0)	0.302	0 (0 to 0)	0.692	0 (0 to 1)	1.000	0 (0 to 0)	0.302
Quinoxyfen	0	2 (0 to 13)				0 (0 to 2)		1 (0 to 10)		18 (2 to 27)		0 (0 to 1)		2 (0 to 11)	
	5	5 (1 to 99)	0.707			0 (0 to 1)	1.000	0 (0 to 3)	1.000	35 (13 to 109)	0.630	0 (0 to 2)	1.000	3 (0 to 14)	1.000
	40	1 (0 to 7)	1.000			0 (0 to 0)	0.692	0 (0 to 7)	1.000	72 (7 to 197)	0.492	0 (0 to 2)	1.000	3 (0 to 8)	1.000
	320	1 (0 to 3)	1.000			0 (0 to 2)	1.000	1 (0 to 25)	1.000	8 (0 to 16)	0.212	0 (0 to 1)	1.000	2 (0 to 10)	1.000
	2,560	6 (0 to 15)	1.000			0 (0 to 1)	1.000	1 (0 to 25)	1.000	15 (0 to 51)	2.795	0 (0 to 4)	1.000	3 (0 to 7)	1.000
Tebuconazole	0	4 (0 to 35)						10 (2 to 113)		17 (3 to 43)				6 (1 to 15)	
	1	3 (1 to 23)	1.000					8 (1 to 17)	1.000	9 (2 to 65)	1.000			4 (1 to 59)	1.000
	5	3 (0 to 56)	1.000					5 (1 to 123)	1.000	4 (0 to 38)	0.634			3 (0 to 14)	0.487
	50	2 (0 to 11)	1.000					15 (4 to 114)	1.000	3 (1 to 36)	0.382			0 (0 to 1)	**0.007**
	500	0 (0 to 2)	**0.031**					109 (8 to 191)	0.385	0 (0 to 2)	**0.007**			0 (0 to 0)	**0.004**
Fungicide	0	0 (0 to 18)		32 (0 to 79)				5 (0 to 19)		30 (0 to 291)				2 (0 to 8)	
mixture	6	0 (0 to 14)	1.000	3 (0 to 137)	0.992			0 (0 to 48)	1.000	19 (0 to 43)	1.000			0 (0 to 26)	1.000
	60	0 (0 to 0)	0.302	2 (0 to 29)	0.212			0 (0 to 12)	0.406	30 (1 to 59)	1.000			0 (0 to 2)	0.054
	600	0 (0 to 0)	0.302	0 (0 to 7)	**0.031**			0 (0 to 0)	**0.045**	0 (0 to 2)	**0.025**			0 (0 to 0)	**0.015**
	3,000	0 (0 to 0)	0.302	0 (0 to 0)	**0.015**			0 (0 to 0)	**0.045**	0 (0 to 1)	**0.025**			0 (0 to 0)	**0.015**

Table S9 continued.

Experiment	Concentration (µg/L)	*H. lugdunensis*		*M. pluvioriens*		*S. aurantiaca*		*T. marchalianum*		*T. angulatum*		*T. myrti*	
		Spores	*P*-value	Spores	*P*-value	Spores	*P*-value	Spores	*P*-value	Spores	*P*-value	Spores	*P*-value
Azoxystrobin	0					46 (16 to 177)		421 (60 to 890)		22 (4 to 100)		0 (0 to1)	
	20					1 (0 to 33)	**0.027**	391 (122 to 3955)	1.000	56 (7 to 196)	1.000	0 (0 to 0)	1.000
	100					0 (0 to 1)	**0.007**	834 (194 to 2070)	1.000	25 (1 to 98)	1.000	0 (0 to 0)	1.000
	500					0 (0 to 1)	**0.006**	70 (0 to 404)	0.119	1 (0 to 15)	**0.028**	0 (0 to 0)	1.000
	2,500					0 (0 to 0)	**0.004**	3 (0 to 5)	**0.008**	0 (0 to 1)	**0.007**	0 (0 to1)	1.000
Carbendazim	0					0 (0 to 9)		69 (9 to 2454)		24 (2 to 402)			
	5					2 (0 to 81)	1.000	18 (2 to 117)	0.385	19 (2 to 298)	1.000		
	35					0 (0 to 4)	1.000	53 (0 to 404)	1.000	7 (0 to 37)	0.999		
	245					0 (0 to 0)	0.299	2 (0 to 11)	**0.013**	0 (0 to 0)	**0.004**		
	1,715					0 (0 to 0)	0.299	0 (0 to 0)	**0.004**	0 (0 to 0)	**0.004**		
Cyprodinil	0	0 (0 to 0)		0 (0 to 1)				2 (0 to 162)		0 (0 to 6)			
	8	0 (0 to 0)	not calculable	0 (0 to 2)	1.000			0 (0 to 40)	1.000	0 (0 to 2)	1.000		
	40	0 (0 to 2)	1.000	0 (0 to 0)	1.000			0 (0 to 2)	0.345	0 (0 to 0)	0.302		
	200	0 (0 to 17)	0.692	0 (0 to 1)	1.000			0 (0 to 0)	0.123	0 (0 to 0)	0.302		
	1,000	0 (0 to 16)	0.692	0 (0 to 2)	1.000			0 (0 to 0)	0.123	0 (0 to 0)	0.302		
Quinoxyfen	0					0 (0 to 2)		9 (0 to 150)		5 (0 to 10)			
	5					1 (0 to 3)	1.000	3 (0 to 411)	1.000	1 (0 to 28)	1.000		
	40					0 (0 to 7)	1.000	0 (0 to 70)	1.000	1 (0 to 53)	0.539		
	320					0 (0 to 4)	1.000	1 (0 to 7)	0.854	2 (1 to 9)	1.000		
	2,560					0 (0 to 1)	1.000	1 (0 to 40)	1.000	5 (0 to 37)	1.000		
Tebuconazole	0					121 (16 to 563)		313 (84 to 1722)		43 (4 to 236)			
	1					41 (19 to 74)	1.000	219 (34 to 1113)	1.000	58 (16 to 135)	1.000		
	5					58 (9 to 365)	1.000	733 (3 to 1161)	1.000	145 (4 to 376)	1.000		
	50					88 (33 to 239)	1.000	301 (3 to 2703)	1.000	13 (1 to 119)	0.803		
	500					78 (27 to 155)	1.000	96 (11 to 1045)	0.212	1 (0 to 4)	**0.010**		
Fungicide	0			0 (0 to 2)		5 (0 to 80)		74 (0 to 279)					
mixture	6			0 (0 to 1)	1.000	7 (0 to 24)	1.000	14 (0 to 36)	0.439				
	60			0 (0 to 3)	1.000	2 (0 to 10)	0.954	97 (0 to 245)	1.000				
	600			0 (0 to 1)	1.000	0 (0 to 1)	0.094	7 (0 to 114)	0.217				
	3,000			0 (0 to 0)	0.687	0 (0 to 0)	**0.045**	0 (0 to 1)	**0.025**				

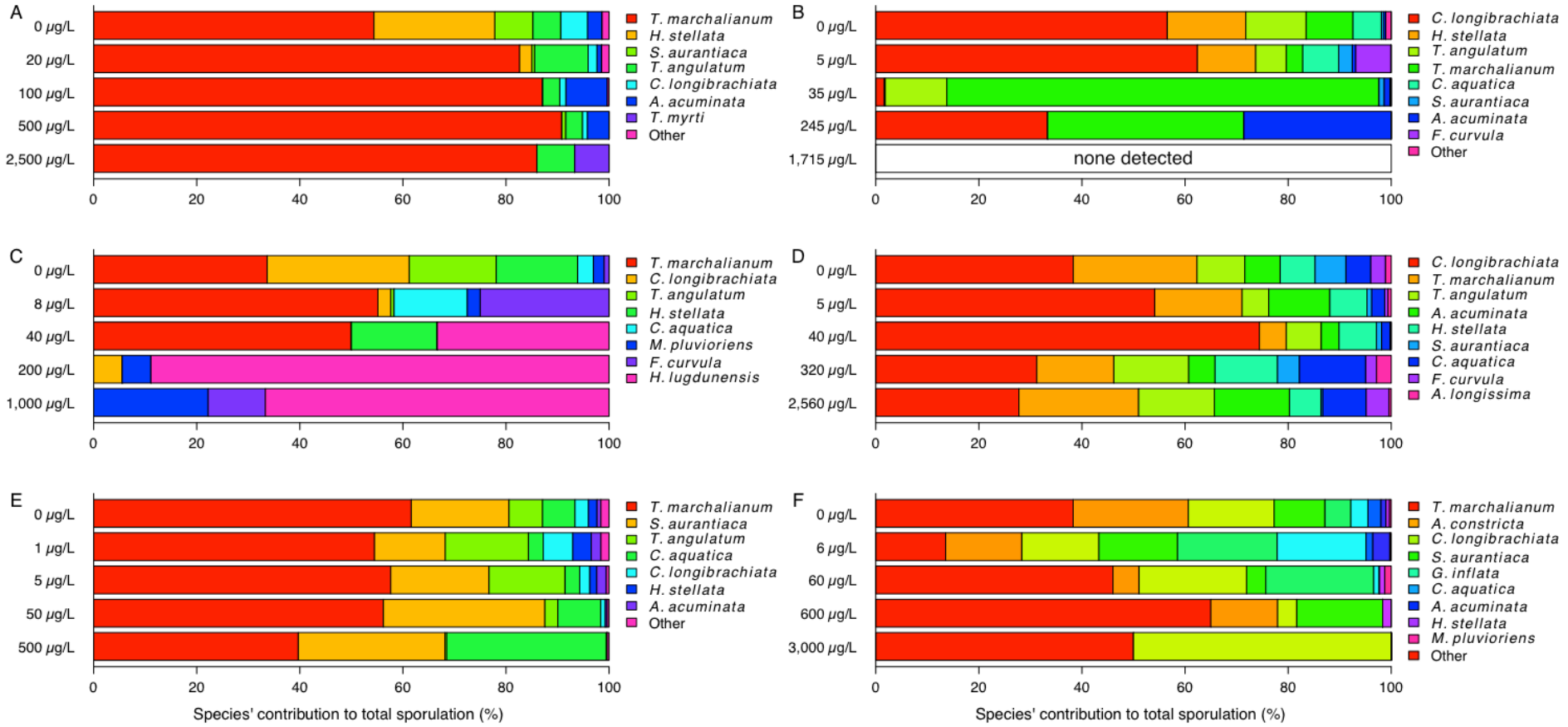

Fig. S1. Mean species' contribution to total aquatic hyphomycete sporulation on leaf discs conditioned in the presence of different concentrations of A) azoxystrobin, B) carbendazim, C) cyprodinil, D) quinoxyfen, E) tebuconazole, and F) the fungicide mixture. All species contributing <1% at all tested concentrations of an experiment are subsumed as "other". Note that each color scheme starts with the species contributing most to sporulation in the respective control.

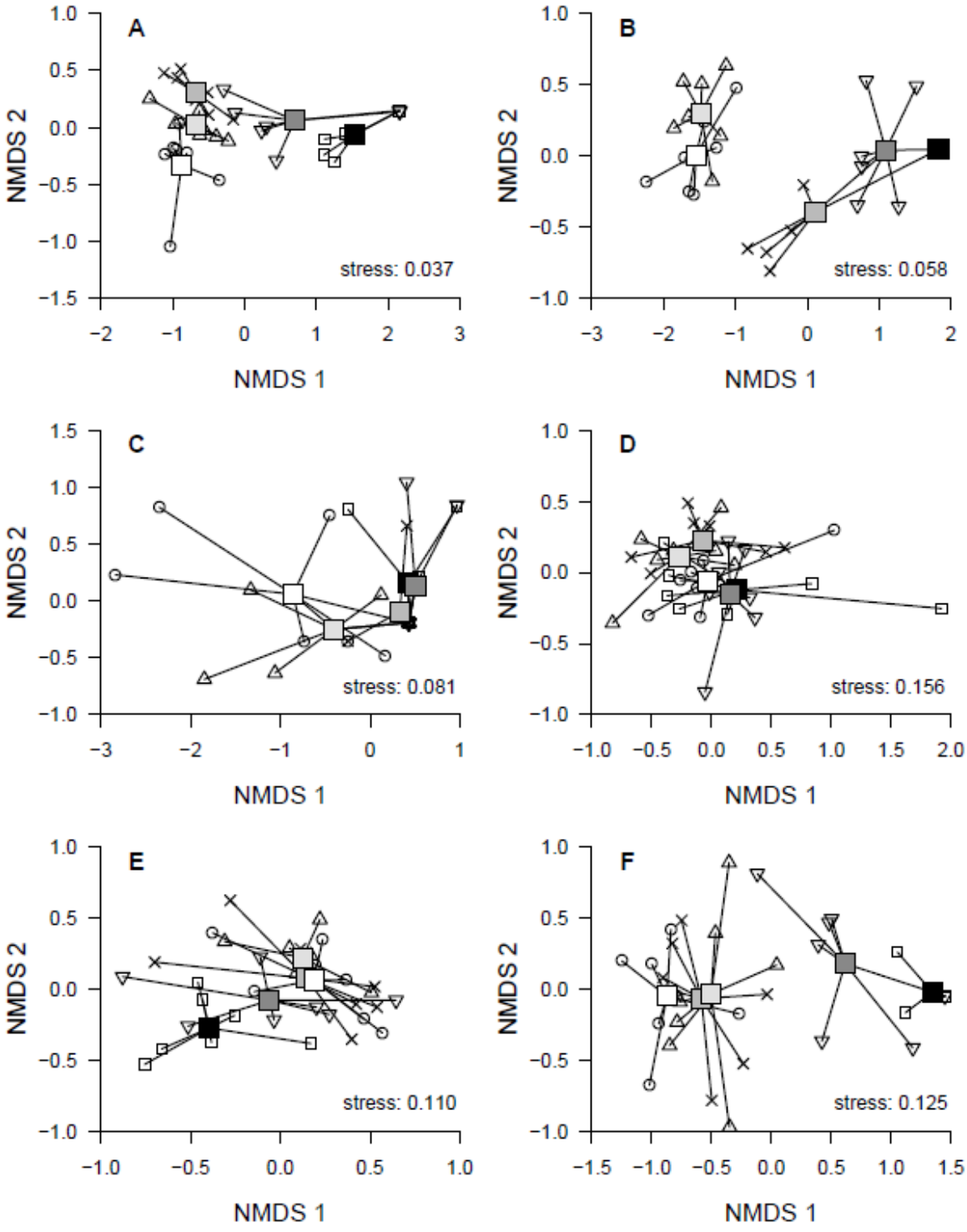

Fig. S2. Non-metric multidimensional scaling (NMDS; an ordination technique to display the dissimilarities of samples; Clarke, 1993) plots of aquatic hyphomycete communities associated with leaf material conditioned in the presence of different concentrations of A) azoxystrobin, B) carbendazim, C) cyprodinil, D) quinoxyfen, E) tebuconazole, and F) the fungicide mixture. Symbols are (from lowest to highest concentration): circles (i.e., control), triangles, crosses, inverted triangles, and squares. Additionally, concentrations (Table 1 of the main document) are indicated by grey scale of the group centroids with white corresponding to the lowest (i.e., control) and black to the highest tested concentration, respectively. Stress values are provided as a measure of "goodness-of-fit" for NMDS, with reasonable fits indicated when below 0.2 (Clarke, 1993).

ASSESSMENT OF MIXTURE EFFECTS

To determine the expected joint effect of the fungicide mixture on the functional endpoints and structure-related sum parameters assessed during the present study, several concentration-response models were fitted to each fungicide's data using the R extension package "drc" (Ritz and Streibig, 2005)). To allow model fitting despite the high variability associated with some endpoints and to circumvent the problems related to the paired data of the functional endpoints, mean or median effect sizes instead of the actual data were used. The model fitting the effect sizes best (based on Akaike's information criterion and expert judgment) was then used to predict the mixture effect using the mixture reference model "independent action", which assumes the mixture components to have dissimilar modes of action (Bliss, 1939). Model formulation for predictions is for instance reported by Backhaus et al. (2000). Both predictions and observations were expressed as percent reduction compared to the respective control. Agreement between model predictions and observations was assumed when the prediction was included in the 95% CI of the observation (*cf.* Rasmussen et al., 2012). Note that the predictions for the two lower mixture concentrations need to be interpreted with caution, since not all fungicides had been tested singly at concentrations as low as applied in the mixture.

The majority of the observed effects either agreed with or were lower than the model predictions (Table S10). Additionally, for fungal biomass instead of the predicted increase in this endpoint significant reductions (zero not included in the 95% CI) were observed (i.e., the effect direction was reversed).

Table S10. Predicted (according to IA) and observed effect sizes (percentage reduction compared to control) for the functional endpoints and structure-related sum parameters assessed during the present study.

Endpoint	Total fungicide concentration in µg/L	Prediction according to IA	Observation (mean or median with 95% CI)	Mixture effect
Gammarids' leaf consumption	6	32.7	5.6 (-28.3 to 38.6)	Agreement
	60	48.2	-0.9 (-41.3 to 43.5)	Lower than predicted
	600	74.1	29.0 (5.7 to 53.7)	Lower than predicted
	3,000	87.8	34.5 (-22.0 to 92.8)	Agreement
Microbial leaf decomposition	6	-8.2	-3.9 (-8.3 to 0.5)	Agreement
	60	1.3	-0.1 (-5.1 to 4.8)	Agreement
	600	19.1	5.6 (1.1 to 10.2)	Lower than predicted
	3,000	32.2	12.1 (7.6 to 16.7)	Lower than predicted
Bacterial density	6	30.6	34.2 (2.9 to 56.8)	Agreement
	60	65.3	11.8 (-27.2 to 43.6)	Lower than predicted
	600	74.2	2.3 (-48.6 to 21.8)	Lower than predicted
	3,000	53.2	-4.6 (-225.9 to 25.7)	Lower than predicted
Fungal biomass	6	-156.9	9.6 (-34.8 to 27.1)	Lower than predicted
	60	-205.0	24.9 (0.8 to 35.0)	Reversed effect direction
	600	-39.9	42.4 (22.4 to 56.1)	Reversed effect direction
	3,000	62.1	54.3 (22.4 to 63.4)	Agreement
Number of fungal species	6	17.3	29.2 (0.6 to 57.7)	Agreement
	60	77.1	27.1 (8.0 to 46.2)	Lower than predicted
	600	97.8	81.3 (71.1 to 91.4)	Lower than predicted
	3,000	99.8	95.8 (89.3 to 100.0)	Agreement
Number of fungal spores	6	-41.3	42.2 (-39.1 to 42.2)	Lower than predicted
	60	92.6	-16.7 (-93.0 to 83.2)	Lower than predicted
	600	100.0	94.6 (31.1 to 100.0)	Agreement
	3,000	100.0	100.0 (99.5 to 100.0)	Agreement

STRUCTURAL DIFFERENCES AMONG CONTROL COMMUNITIES

Table S11. *P*-values for comparisons among control communities for all structural endpoints in the order number of fungal species and fungal spore production (both assessed with Wilcoxon rank-sum tests with Bonferroni adjustment), fungal community composition (PERMANOVA with Bonferroni adjustment), fungal biomass (Tukey's test), and bacterial density (Wilcoxon rank-sum tests with Bonferroni adjustment). All *P*-values <0.05 are printed in bold. In addition, the date of retrieval of inocula used in the experiments is indicated.

	Azoxystrobin - Sep-2012	Carbendazim - Apr-2013	Cyprodinil - Jan-2013	Quinoxyfen - Mar-2013	Tebuconazole - Jul-2012
Carbendazim Apr-2013	1.000/1.000/**0.030**/0.961/1.000	-	-	-	-
Cyprodinil Jan-2013	**0.041**/0.166/**0.015**/**0.014**/1.000	0.088/0.0612/0.075/**0.001**/1.000	-	-	-
Quinoxyfen Mar-2013	0.082/**0.0175**/**0.030**/0.096/1.000	0.603/**0.009**/**0.015**/0.373/1.000	0.494/1.000/0.195/**<0.001**/1.000	-	-
Tebuconazole Jul-2012	1.000/1.000/0.945/0.996/1.000	0.418/1.000/**0.015**/0.999/0.170	**0.031**/1.000/**0.045**/**0.003**/0.390	**0.035**/**0.0175**/**0.045**/0.217/1.000	-
Mixture Jul-2013	0.452/0.2622/**0.015**/**<0.001**/1.000	1.000/**0.0175**/**0.030**/**<0.001**/1.000	0.099/1.000/**0.030**/0.590/1.000	1.000/0.105/**0.015**/**<0.001**/1.000	0.168/0.7955/0.060/**<0.001**/1.000

REVIEW OF RISK ASSESSMENT DOCUMENTS OF APPROVED FUNGICIDES

The EU Pesticides Database for active substances (EC, 2014) was accessed on April 12, 2014 and a list of all fungicides approved in the European Union was extracted. These 134 substances were assigned to modes of action (FRAC, 2014) and the respective “conclusion on pesticide review” by the European Food Safety Authority (EFSA) or – if the former was not available – the review report by the European Commission’s Directorate General for Health & Consumers was downloaded. To allow inferences for the specific group of substances assessed during the present study, inorganic fungicides (e.g., copper compounds), natural substances, organisms, and substances only approved for indoor use were excluded from the final analyses, resulting in 98 relevant fungicides.

The risk assessment documents were screened for the effect assessment methods used and the derived regulatory acceptable concentrations (RACs). The explicit mentioning of the use of methods listed by EFSA (2013; i.e., the geomean approach, species sensitivity distributions, refined exposure laboratory tests, micro- or mesocosm studies, lowering the assessment factor, and replacing the most sensitive species by a less sensitive one) was considered higher-tier effect assessment. This resulted in 22 fungicides, which had been assessed at a higher tier. For these substances, also a first-tier RAC was calculated using all available single species data. This, however, was only possible when an EFSA conclusion was available (15 fungicides) since the European Commission did not explicitly mention the final choice of endpoints and assessment factors for risk assessment in their review reports. Following this approach, RACs, which are intended to be protective thresholds for populations of aquatic organisms (EFSA, 2013), were increased by higher-tier effect assessment by a maximum and median factor of 42.2 and 9.6 (95% CI from 2.3 to 18.6), respectively.

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APPENDIX A.4

The relative importance of diet-related and waterborne effects of copper for a leaf-shredding invertebrate

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ABSTRACT

Copper (Cu) exposure can increase leaf-associated fungal biomass, an important food component for leaf-shredding macroinvertebrates. To test if this positive nutritional effect supports the physiological fitness of these animals and to assess its importance compared to waterborne toxicity, we performed a 24-day-bioassay in combination with a 2x2 factorial design using the amphipod shredder *Gammarus fossarum* and a field-relevant Cu concentration of 25 µg/L (*n*=65). Waterborne toxicity was negligible, while gammarids fed leaves exposed to Cu during microbial colonization exhibited a near-significant impairment in growth (~30%) and a significantly reduced lipid content (~20%). These effects appear to be governed by dietary uptake of Cu, which accumulated in leaves as well as gammarids and likely overrode the positive nutritional effect of the increased fungal biomass. Our results suggest that for adsorptive freshwater contaminants dietary uptake should be evaluated already during the registration process to safeguard the integrity of detritus-based ecosystems.

KEYWORDS

dietary exposure – environmental risk assessment – leaf litter breakdown – physiological fitness – shredder

INTRODUCTION

Copper (Cu) is an essential trace element to terrestrial and aquatic organisms, serving *inter alia* as a component of a variety of enzymes (e.g., the oxygen-carrier hemocyanin in crustaceans; Flemming and Trevors, 1989). In freshwater ecosystems, however, human activities such as mining and agriculture have led to elevated Cu-levels (e.g., Sridhar et al., 2000; Süss et al., 2006). As a result, Cu poses a high risk to aquatic life (e.g., Donnachie et al., 2014). Accordingly, numerous studies have focused on adverse effects of Cu on individual species up to whole freshwater food webs (see e.g. reviews by Eisler, 1998; Flemming and Trevors, 1989; Nor, 1987). However, implications of Cu in the colonization of leaf material by aquatic fungal decomposers (i.e., conditioning) and the resulting effects on the nutrition of leaf-shredding invertebrates, which rely heavily on the changes in leaf quality brought about by fungi (Bärlocher, 1985; Suberkropp, 1992), have largely been ignored. In this context, it was recently shown that Cu exposure can increase

leaf-associated fungal biomass, which was sensed by the amphipod shredder *Gammarus fossarum* KOCH (a species known to prefer food that allows high growth rates; Bärlocher and Kendrick, 1973), actively selecting Cu exposed over unexposed leaves (Zubrod et al., 2015b). It may hence be hypothesized that the presence of Cu during the conditioning of leaf material positively affects the physiological status of shredders over the long term (cf. Bärlocher and Kendrick, 1973), especially as metal toxicity via dietary uptake is commonly considered comparably insignificant (Abel and Bärlocher, 1988; Sridhar et al., 2001).

To test this hypothesis as well as its importance compared to waterborne toxicity, we employed a 24-day-bioassay in combination with a 2x2 factorial design: the first factor was the absence or presence of Cu during the conditioning of leaf material (used as food for our model shredder) and the second factor was the absence or presence of Cu in the medium used for culturing the shredders during the bioassay (=waterborne exposure). Despite its high baseline Cu content (e.g. Bourgeault et al., 2013), we chose *G. fossarum* as model shredder as it is a key species in leaf liter decomposition in many European streams (Dangles et al., 2004) and its effect thresholds in terms of Cu related feeding preferences and waterborne Cu toxicity are well established (Zubrod et al., 2014; Zubrod et al., 2015b). On this basis, we expected a Cu concentration of 25 µg/L, which is realistic for instance for streams impacted by wastewater treatment plant effluents or agriculture (e.g., da Silva Oliveira et al., 2007; Süss et al., 2006), to increase leaf-associated fungal biomass (cf. Zubrod et al., 2015b). We hypothesized further that, in consequence of long-term consumption of such Cu exposed leaves, the physiological fitness (judged by growth and lipid content) of *Gammarus* would increase. Since waterborne toxicity at the applied Cu concentration should be low (cf. Zubrod et al., 2014), an overall positive net effect on *Gammarus* was expected when subjected to combined effects from ingestion of Cu exposed leaves and exposure via water.

MATERIALS AND METHODS

SOURCES OF LEAVES, MICROORGANISMS, AND GAMMARIDS

Leaves of *Alnus glutinosa* (black alder), a species that features comparably low C:N ratios of <20 (e.g. Foucreau et al., 2013) and is decomposed in freshwater systems relatively fast (e.g. Schindler and Gessner, 2009), were collected in October 2012 near Landau, Germany (49°11'N; 8°05'E) and stored at -20°C until further

processing. Following the procedure described by Zubrod et al. (2011), leaves were deployed for 14 days in fine-mesh bags in the Rodenbach, Germany (49°33'N, 8°02'E), upstream of any agricultural activity, settlement, and wastewater inlet, to establish a natural microbial community. Back in the laboratory, unconditioned (i.e., previously not subjected to microbial colonization) leaves were added to the retrieved leaf material and the mixed leaves were kept in aerated conditioning medium (Dang et al., 2005) at 16±1°C in total darkness for at least another 12 days before use as microbial inoculum.

Seven days prior to their introduction into the bioassay, *G. fossarum* were kick-sampled in the Hainbach, Germany (49°14'N; 8°03'E), upstream of any agricultural activity, settlement, and wastewater inlet, where the local population is exclusively composed of cryptic lineage B (Feckler et al., 2014). Only visually non-parasitized adult males (6 to 8 mm body length) were used. Throughout their acclimation to laboratory conditions, animals were kept in a temperature-controlled chamber at 16±1°C in total darkness and fed *ad libitum* with pre-conditioned black alder leaves, while they were gradually adapted to the nutrient medium used during the bioassay (i.e., SAM-5S; Borgmann, 1996).

EXPERIMENTAL DESIGN

Our experimental design, following in general Zubrod et al. (2011), consisted of two compartments – a leaf conditioning to provide gammarids with microbially colonized food and the bioassay with *Gammarus* (see below). The employed 2x2 factorial design, resulted in four treatments: (i) a control with uncontaminated leaf material and gammarids cultured in Cu free medium, (ii) leaf material exposed to Cu, gammarids not (i.e., indirect effect pathway), (iii) gammarids exposed to Cu, leaf material not (i.e., direct effect pathway), and (iv) leaf material and gammarids exposed to Cu (i.e., both pathways combined). All Cu containing media were dosed with Cu sulfate pentahydrate (Fluka, St. Gallen, Switzerland) at a nominal concentration of 25 µg Cu/L.

EXPERIMENTAL CONDITIONING

Leaf strips of 3 to 5 cm x 5 to 9 cm were cut from unconditioned black alder leaves and placed in aerated circular aquaria (*n*=3; 150 strips per aquarium) filled with 14 L conditioning medium together with 50 g fresh weight of the microbial inoculum.

Conditioning took place at 16±1°C in total darkness. Three aquaria were dosed with Cu at 25 µg/L, while three represented control conditions. The conditioning medium (with the appropriate concentration of Cu) was renewed every 3 days to ensure a continuous exposure, accounting *inter alia* for losses due to potential adsorption to the test vessels (cf. Zubrod et al., 2014). After 12 days, two leaf discs (2.0 cm diameter) were cut from each strip, one disc from each side of the leaf's midrib. Discs were immediately introduced into the bioassay or frozen (-20°C) for the analyses of leaf-associated fungal biomass and Cu content. To provide the gammarids during the bioassay every 6 days with food of constant quality, four conditionings were performed, each one starting 12 days before the respective leaves were introduced into the bioassay.

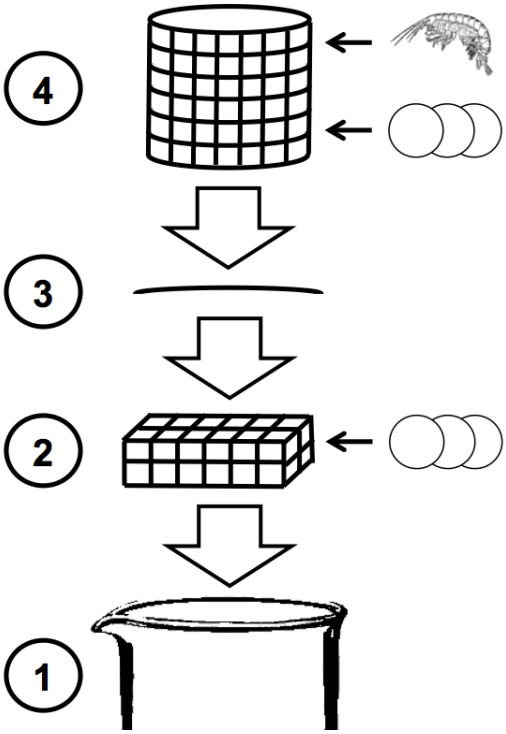

Fig. 1. Scheme illustrating the setup of the bioassay replicates. At the bottom of a (1) 250-mL glass beaker filled with 200 mL bioassay medium a (2) rectangular cuboid cage containing three leaf discs is situated. This cage is topped by a (3) watch glass on which a (4) cylindrical cage containing another three leaf discs and one gammarid is placed. For more details see text.

BIOASSAY

Each of the four treatments of the bioassay comprised 65 replicates consisting of 250-mL glass beakers filled with 200 mL bioassay medium (item 1 in Fig. 1). Beakers were equipped with cylindrical cages made from stainless steel mesh screen and

within each cage one gammarid was placed together with three leaf discs originating from three separate leaf strips (item 4 in Fig. 1). These cages allowed a careful transfer of the animals to new vessels containing fresh bioassay medium (with the appropriate concentration of Cu) every 3 days (ensuring a continuous exposure; cf. Zubrod et al., 2014) and prevented the animals from coprophagy. In addition, a rectangular cuboid cage made from the same stainless steel mesh screen (item 2 in Fig. 1) was situated on the bottom of each glass beaker below the cylindrical one. This cage contained the corresponding three leaf discs, which originated from the same three leaf strips as the leaf discs offered as food to the gammarid and allowed to control for abiotic and microbial leaf mass losses in each replicate. To prevent that the feces produced by the animal interacts with the leaf discs within the rectangular cuboid cage, the two cages were separated by a watch glass (item 3 in Fig. 1). During the bioassay, glass beakers were continuously aerated and situated in a temperature-controlled chamber at 16±1°C in total darkness.

At every second renewal of the bioassay medium also the leaf discs were replaced (i.e., every 6 days). At the same time, remaining leaf discs and any leaf tissue shredded off were removed from the glass beakers to quantify gammarids' leaf consumption by separately drying the leaf material from both cages at 60°C for 24 h and weighing them to the nearest 0.01 mg. Moreover, to assess gammarids' feces production, old bioassay medium was filtered through pre-weighed glass fiber filters (GF/6, Whatman, Dassel, Germany). One filter was used to collect the feces produced by one gammarid over 6 days, i.e., for two consecutive medium renewals, and the filters were stored at 60°C in between these renewals. To feature exclusively fecal pellets, filters were carefully checked for leaf fragments before they were dried and weighed as described above. The removed leaf fragments were added to the recovered leaf material of the respective replicate and point in time to allow an accurate quantification of leaf consumption and feces production. To control for increases in filter weight due to microbial decomposition of leaf material, three additional replicates per treatment were set up without gammarids. Mortality of gammarids was checked during medium renewals but was – with a maximum of 3% after 24 days – inconspicuous. At the termination of the experiment, surviving animals were carefully blotted dry using clean tissues, shock-frozen in liquid nitrogen, and preserved individually in glass tubes at -75°C (see for an overview of the timeline of events during the bioassay Table S1 in the Supplementary Information).

QUANTIFICATION OF LEAF-ASSOCIATED FUNGAL BIOMASS

Composite samples from five leaf strips (*n*=12; three replicate aquaria x four conditioning runs) were analyzed for their ergosterol content as a proxy for fungal biomass according to Gessner (2005). After extraction in alkaline methanol and purification by solid-phase extraction (Sep-Pak® Vac RC tC18 500 mg sorbent, Waters, Milford, MA, USA), ergosterol was quantified by high-performance liquid chromatography (1200 Series, Agilent Technologies, Santa Clara, CA, USA) using a LiChrospher® 100 RP 18-5 m column (250.0 mm x 4.6 mm, particle size 5 mm, CS-Chromatographie Service, Langerwehe, Germany).

QUANTIFICATION OF GAMMARIDS' LIPID CONTENT

The lipid content of 12-14 lyophilized and weighed (to the nearest 0.01 mg) animals per treatment was analyzed following the method described by Van Handel (1985) and modified by Zubrod et al. (2011) for use with a microplate reader (Tecan Infinite® M200, Tecan Group, Crailsheim, Germany). Lipids were extracted in 1:1 chloroform:methanol (v:v), before reaction with sulfuric acid and vanillin-phosphoric acid reagent. For quantification, absorbance at 490 nm was measured and read against a standard curve prepared from commercially available soybean oil (Sojola Soja-Öl, Vandemoortele, Herford, Germany).

CU CONCENTRATIONS IN MEDIA, LEAF MATERIAL, AND GAMMARIDS

Cu concentrations were measured in fresh and 3-day-old (i.e., before medium renewal) conditioning and bioassay medium (*n* = 4) using inductively coupled plasma quadrupole mass spectrometry (Rosenfeldt et al., 2014). Results of the measurements justified an accurate Cu dosage (deviation from nominal ≤7%; Table 1). To assess the potential for trophic transfer of Cu along the detrital food chain, Cu was quantified in four composite leaf samples. These originated from leaf strips preserved after conditioning, which might slightly underestimate the true leaf Cu contents within the bioassay due to additional exposure. Moreover, three individual gammarids (preserved at the termination of the bioassay) per treatment were analyzed. Lyophilized samples were weighed and samples as well as blanks and the reference material GBW 07602 (Institute of Geophysical and Geochemical Exploration, China) were digested for 4 days at gradually increased temperatures

(30-80°C) in aqua regia. Afterwards, all samples were filled up with diluted nitric acid (1%) to a total volume of 10 mL and measured like the liquid samples.

Table 1. Mean (*n* = 4; with 95% CI) Cu concentrations measured in fresh or 3-day-old (collected prior to medium renewal) conditioning and bioassay medium.

Compartment	Treatment	Fresh (µg Cu/L)	3-day-old (µg Cu/L)
Conditioning	Control	0.5 (0.3-0.8)	1.4 (0.8-2.1)
	Cu exposure	26.2 (17.3-35.0)	5.7 (2.9-8.6)
Bioassay	Control	0.6 (0.0-1.4)	0.7 (0.0-1.7)
	Indirect	0.5 (0.0-1.1)	0.8 (0.4-1.2)
	Direct	23.3 (20.2-26.3)	9.9 (2.3-17.4)
	Combined	24.0 (21.2-26.8)	11.4 (0.0-23.5)

DATA ANALYSIS

Leaf consumption and feces production of the gammarids were calculated as per Zubrod et al. (2011) and observations made on each animal were averaged to display the treatments' effects over the whole experimental duration. Animals' growth (in terms of dry weight gain) during the bioassay was determined by subtracting the mean dry weight of 66 lyophilized gammarids from the start of the bioassay from the final dry weight (after lyophilization) of the bioassay animals.

Data were visually checked for normality and Levene's test was used to assess homoscedasticity. For all gammarid-related endpoints, two-way ANOVAs were either performed on the original data or, if the presumptions for parametric testing were violated, on ranks. Due to heteroscedasticity, leaves' fungal biomass and Cu content were assessed using Welch's *t*-tests. R version 3.1.1 for Mac (R Development Core Team, 2014) was used for all statistics and figures.

RESULTS AND DISCUSSION

INDIRECT AND DIRECT EFFECTS OF CU IN GAMMARUS

As expected (cf. Zubrod et al., 2015b), the presence of Cu during microbial conditioning resulted in a significantly increased leaf-associated fungal biomass (by ~50%, $P = 0.041$, Fig. 2a) compared to the control. Given that the nutritional value of microbial biomass is usually four to ten times higher than that of the leaf substrate (Bärlocher and Kendrick, 1975), a higher food quality of leaves exposed to Cu can be presumed. Moreover, Cu exposure was recently shown to alter the community composition of leaf-associated fungi, while tending to favor species preferred by *Gammarus* (Zubrod et al., 2015b). Accordingly and in line with our hypotheses, the

significantly lower leaf consumption of *G. fossarum* triggered via the indirect effect path (by ~10%, Table 2, Fig. 3) could thus be interpreted as a mechanism to account for a higher food quality since less food is required to meet the animals' nutritional needs (cf. Simpson and Simpson, 1990). However, the near-significant reduction in gammarid growth and a significant decrease in lipid content (~30% and ~20%; Table 2, Fig. 4a,b) indicate an impaired physiological status of animals subjected to this effect pathway, which does not comply with the expectations for consumption of a preferred food source (cf. Bärlocher and Kendrick, 1973).

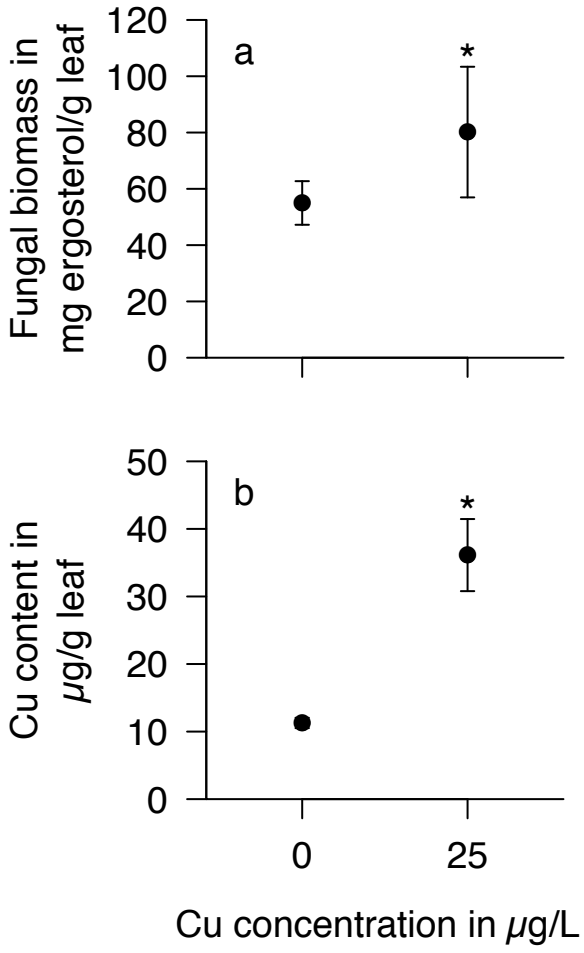

Fig. 2. Means with 95% CIs of (a) fungal biomass (*n*=12) and (b) Cu content (*n*=4) per g dry weight of leaf material exposed to 0 (i.e., control) or 25 µg Cu/L during microbial conditioning. Asterisks denote statistically significant differences.

In this context, toxicity arising from the re-dissolution of Cu from exposed leaf material seems unlikely to be the underlying mechanism since aqueous Cu concentrations in vessels containing exposed discs were not elevated (Table 1). Instead, these observations suggest that toxic effects due to dietary Cu uptake may

have overridden the positive nutritional effect related to the increased leaf-associated fungal biomass. In line with this, the presence of Cu during conditioning led to a significant increase in the Cu content of exposed leaf material (by more than 200%, $P < 0.001$, Fig. 2b). This was probably driven by a combination of adsorption of the metal to the leaf substrate and the associated microorganisms as well as Cu accumulation in these microbes and the extracellular polymeric substances they produce (as reviewed by Schaller et al., 2011b). As a consequence, the leaves' Cu content increased from about 10 µg/g, which is well in the range of Cu background levels in leaves reported in the literature (Golobic et al., 2012; Sridhar et al., 2001), to about 40 µg/g. While the former Cu content was shown to satisfy the dietary requirements of a marine decapod, the latter was above its optimal range and resulted in a reduced growth (Lee and Shiau, 2002). This gives – despite potential differences in the sensitivity towards Cu between marine and freshwater crustaceans – additional support to the assumption of dietary uptake as trigger for the physiological effects observed during this study (Fig. 4a).

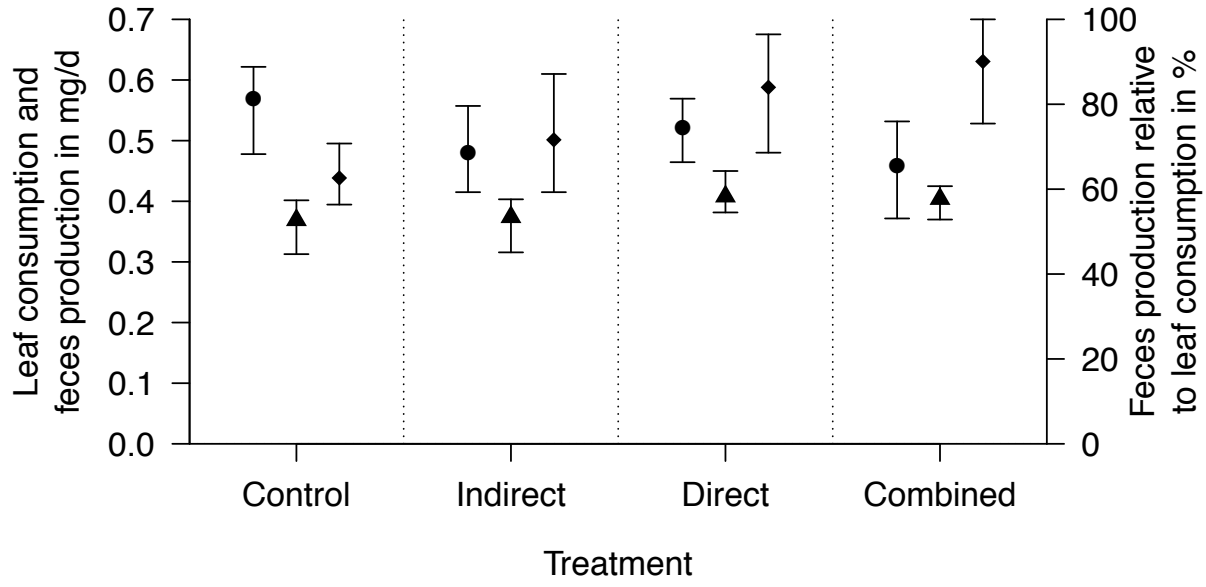

Fig. 3. Medians with 95% CIs of leaf consumption (points) and absolute (triangles) and relative (diamonds) feces production of *G. fossarum* being subjected for 24 days to four treatments ($n \geq 61$): a Cu free control, gammarids receiving leaves conditioned in the presence of Cu (i.e., indirect), gammarids being directly exposed to Cu (i.e., direct), and a combination of the indirect and direct treatments. For statistical evaluation see Table 2.

Accordingly, the Cu content of animals subjected to the indirect path was significantly increased (Table 2; Fig. 4c), which seems to be only slightly influenced by the presence of Cu containing food in the animals' guts (see calculations in

Table 2. ANOVA-tables for all gammarid-related endpoints. All *P*-values <0.05 are printed in bold.

Endpoint	Source of variation	d.f.	SS	MS	*F*	*P*
Leaf consumption	Indirect	1	20529	20529	3.983	**0.047**
	Direct	1	3124	3124	0.606	0.437
	Indirect x Direct	1	1	1	0.000	0.989
	Residuals	245	1262836	5154		
Feces production (absolute)	Indirect	1	1731	1731	0.345	0.557
	Direct	1	53154	53154	10.591	**0.001**
	Indirect x Direct	1	2026	2026	0.404	0.525
	Residuals	245	1229570	5019		
Feces production (relative)	Indirect	1	9158	9158	1.845	0.176
	Direct	1	59603	59603	12.005	**0.001**
	Indirect x Direct	1	1371	1371	0.276	0.600
	Residuals	245	1216367	4965		
Growth	Indirect	1	2.14	2.145	3.242	0.073
	Direct	1	0.02	0.015	0.023	0.880
	Indirect x Direct	1	0.00	0.001	0.001	0.972
	Residuals	244	161.42	0.662		
Lipid content	Indirect	1	11944	11944	5.150	**0.028**
	Direct	1	97	97	0.042	0.839
	Indirect x Direct	1	98	98	0.042	0.838
	Residuals	48	111333	2319		
Cu content	Indirect	1	211.7	211.7	23.18	**0.001**
	Direct	1	846.7	846.7	92.71	**<0.001**
	Indirect x Direct	1	385.3	385.3	42.19	**<0.001**
	Residuals	8	73.1	9.1		

Supplementary Information). Moreover, the direct path even resulted in a significantly more pronounced increase (by ~50%) compared to the indirect one (by ~30%; Student's *t*-test; $P = 0.012$), which may partly be due to Cu adsorption to the animals' cuticle (Schaller et al., 2011a). Nonetheless, waterborne Cu exposure did not impair animals' physiological status (Table 2; Fig. 4a,b). The most likely explanation for this absence of physiological effects in animals exposed via water seems to be that Cu from waterborne and dietary exposures results in differing modes of toxic action, which is supported by a recent study by Hook et al. (2014) using the estuarine amphipod *Melita plumulosa*: Only waterborne exposure resulted in an increased expression of transcripts linked with the cytoskeleton – potentially in response to oxidative stress; chitinase and digestive protease transcript expression levels, in contrast, were decreased particularly by dietary exposure. In crustaceans, reduced

activities of the latter group of enzymes have been linked to decreases in consumption and growth (Dedourge-Geffard et al., 2009 and references therein). These mechanisms match well with the decreases in gammarids' leaf consumption and growth observed in response to the dietary uptake of Cu in the present study (Figs. 3 and 4a).

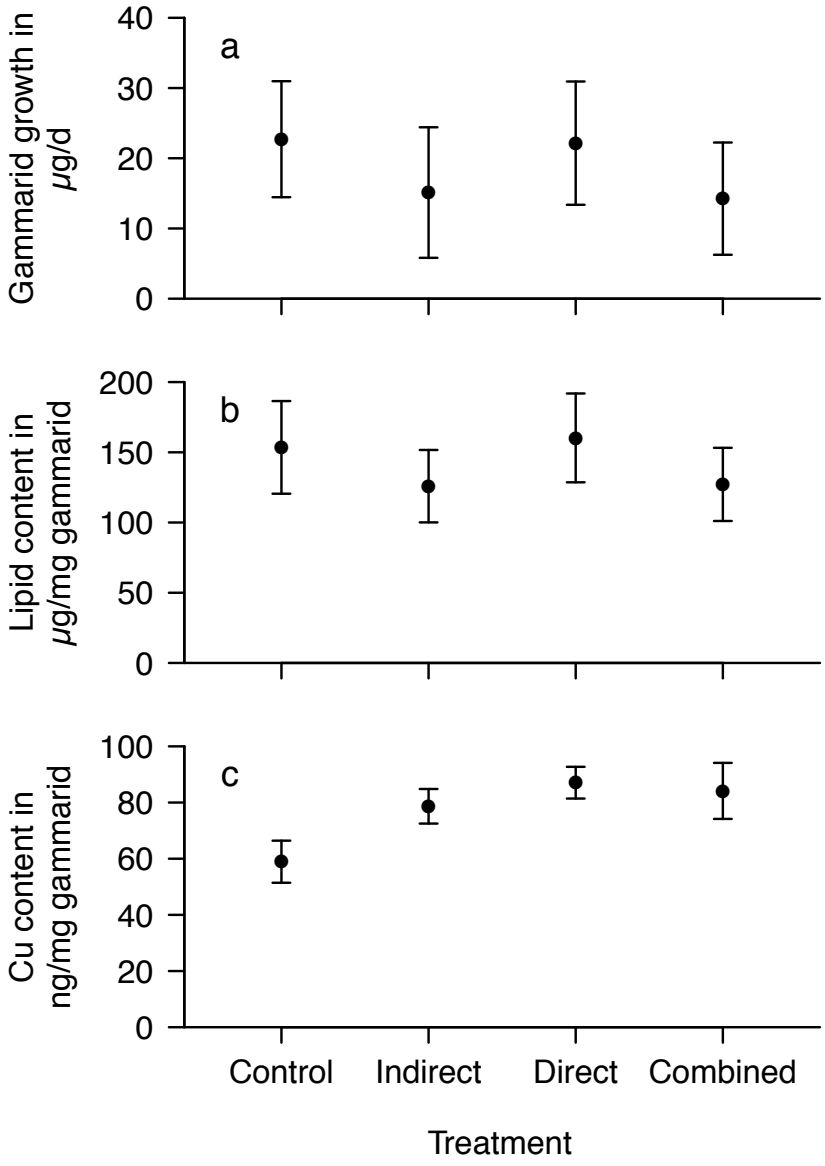

Fig. 4. Means with 95% CIs of (a) growth (n≥61) as well as (b) lipid (n≥12) and (c) Cu content (n=3) of *G. fossarum* being subjected for 24 days to four treatments: a Cu free control, gammarids receiving leaves conditioned in the presence of Cu (i.e., indirect), gammarids being directly exposed to Cu (i.e., direct), and a combination of the indirect and direct treatments. For statistical evaluation see Table 2.

Another plausible explanation could be that the mechanisms available in crustaceans for protection against Cu toxicity, i.e., sequestration in the hepatopancreas as non-

bioavailable complexes and excretion via feces (as reviewed by Schaller et al., 2011b), may be less effective for dietary exposure. The significant increase in absolute feces production (by ~10%; Table 2; Fig. 3) due to the direct effect pathway, which was not observed for the indirect path could be interpreted as support for this explanation. However, when displayed relative to the ingested food (Fig. 3), also the indirect path tended to increase feces production (~10%; although not statistically significant; Table 2). Moreover and as described for grass shrimps (Lee and Shiau, 2002) and terrestrial isopods (Golobic et al., 2012), *G. fossarum* showed a limited capacity to accumulate Cu with maximum body burdens of approximately 85 ng/mg at the termination of the experiment (Fig. 4c). As the combination of exposure via food and water did not result in an additive body burden (significant interaction term of 2x2 ANOVA in Table 2) this capacity seems pathway-independent. As a consequence, the different effect patterns uncovered as a result of dietary and waterborne exposure may not be related to the effectiveness to regulate total Cu accumulation. In contrast, the pathway-dependent Cu distribution within the animals and the resulting activity of Cu in different organs may trigger the differences in transcript expression levels and ultimately the physiological changes observed here.

IMPLICATIONS FOR STREAM ENERGY FLOW

Our results indicate that field-relevant Cu concentrations as low as 25 µg/L may have detrimental implications in detritus-based stream food webs via both dietary and waterborne exposure of shredders. For instance, since gammarids require lipids for reproduction (Glazier, 2000; Plaistow et al., 2003), the observed lower lipid content may be indicative for a lower reproductive capacity (Koop et al., 2011), which may finally result in a lower abundance and in consequence lower contribution of *Gammarus* to local leaf breakdown.

Furthermore, even though feces is generally considered a high quality food resource in stream food webs (Cummins and Klug, 1979), particularly the absolute increase in feces production triggered by the direct effect pathway (Fig. 3) seems worrying. Both food uptake (Fig. 3) and physiological status (Figs. 4a,b) of animals subjected to this pathway were unaffected, indicating a nutrient and energy assimilation from the ingested food equal to or above the control level (to counteract the potentially increased energy demand to cope with Cu stress; Maltby, 1999). As a consequence, the same – or a lower – amount of unassimilated energy and nutrients would be

egested via a higher amount of feces indicating a decreased energy and nutrient concentration and thus an impaired nutritional quality of the feces. This highlights a need for mechanistic studies on chemical stressors' effects on the nutritional quality of animal-derived fine particulate organic matter (cf. Bundschuh and McKie, under review). Moreover, given that crustaceans excrete excess metals via feces (as reviewed by Schaller et al., 2011b), the feces of animals subjected to Cu exposure via food and water may contain high concentrations of this metal (cf. Weeks and Rainbow, 1994). These concentrations may even further increase after egestion due to the high surface area of fecal pellets available for adsorption as well as accumulation by associated biofilms (Schaller et al., 2010), which may at least partly explain the particularly high metal concentrations that can be found in the fine particulate organic matter fraction in contaminated streams (Watanabe et al., 2008). This renders dietary exposure of collecting species likely (Bundschuh and McKie, under review), which seems all the more worrying given the presumed lower nutrient content of fecal pellets produced due to Cu exposure and the potentially elevated consumption of these particles by collectors to cover nutritional needs.

In addition, gammarids represent a key prey for instance for a variety of predatory fish species (e.g., MacNeil et al., 1999). As both exposure via food and water resulted in a significantly increased Cu body burden in *Gammarus* and it was shown that in fish dietary exposure can be the dominant route of metal uptake (as reviewed by Dallinger et al., 1987), Cu transfer over the detrital food web may finally also affect higher trophic levels.

IMPLICATIONS FOR ENVIRONMENTAL RISK ASSESSMENT

Previous studies on organisms associated with autotrophic food webs, namely algae and daphnids, revealed negative implications of dietary exposure only at very high Cu contents in food (~3,000 µg/g; De Schamphelaere et al., 2007). In contrast, the present study revealed detrimental effects of Cu, which seem to be linked to the uptake via food with a concentration more than 80 times lower (i.e., ~40 µg/g; Fig. 2b) resulting from exposure of leaf material to a low, environmentally realistic water Cu concentration. Thus, it appears sensible to consider the risks associated with dietary exposure already during the registration process of chemical compounds, which seems particularly necessary for detritus-based food webs. However, in the European Union the assessment of dietary exposure is recommended exclusively for

fish (EFSA, 2013), although ecosystem functioning may be critically affected as a result of dietary exposure of organisms at lower trophic levels (cf. Bundschuh et al., 2013). Moreover, testing for dietary exposure is triggered by extremely high octanol/water partition coefficients (log P_{ow} > 6; EFSA, 2013). However, Cu, for instance, does not meet this criterion (FOOTPRINT, 2014) but is nonetheless highly adsorptive. Consequently, other criteria as for instance the K_{OC} value (e.g., as high as 9,500 for Cu sulfate; FOOTPRINT, 2014) may be more suitable indicators of substances' potential to accumulate in food and could be used as triggers for long-term feeding studies involving *inter alia* detritus feeders as suggested here.

CONCLUSION

Contrasting with the common assumption that waterborne exposure to contaminants is the major effect path for detritus feeders (Abel and Bärlocher, 1988; Forrow and Maltby, 2000; Sridhar et al., 2001), we demonstrated that dietary exposure via consumed leaf material may be an even more important route. This seems worrying given that detritus can be a significant sink for metals and organic contaminants (e.g., Bundschuh et al., 2013; Sridhar et al., 2001; Zubrod et al., 2015a). To safeguard the integrity of aquatic ecosystems, it thus seems necessary that prospective risk assessments cover all significant exposure routes including dietary uptake. But also retrospective risk assessments, i.e., chemical monitoring, may gain a more integrative endpoint for an ecosystem's exposure and the associated risks for the inhabiting biota, when measuring potentially occurring moderately to highly adsorptive contaminants in detritus (cf. Bundschuh et al., 2014).

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SUPPLEMENTARY INFORMATION

TIMELINE OF EVENTS DURING THE BIOASSAY

Table S1. Timeline of events during the bioassay indicating on which days gammarids were provided with fresh medium and food, when the four filters per replicate were used to retain produced feces, and when gammarids were preserved for the determination of growth and lipid content.

Day	Fresh medium	Fresh food	Filter #	Preservation
0 (start)	X	X	-	X
3	X	-	1	-
6	X	X	1	-
9	X	-	2	-
12	X	X	2	-
15	X	-	3	-
18	X	X	3	-
21	X	-	4	-
24 (termination)	-	-	4	X

CONTRIBUTION OF GAMMARIDS' GUT CONTENT TO THEIR CU BODY BURDEN

A share of the copper (Cu) body burden in gammarids may arise from their gut contents, namely by the Cu adsorbed to the ingested food. The following calculation shows, by following a worst-case procedure, that this share is negligible for the conclusions presented in the main body of the manuscript.

Assuming a gut passage time of 48 h, which is at the upper end for gammarids reported in literature and likely largely overestimates the gut content (Willoughby and Earnshaw, 1982), the amount of Cu contained in an animal's gut is two times its average daily leaf consumption times the average Cu content of the ingested leaf material. This results in the following gut Cu contents:

- 2 days x 0.57 mg/day x 11.3 ng Cu/mg = 12.9 ng Cu control
- 2 days x 0.48 mg/day x 36.1 ng Cu/mg = 34.7 ng Cu indirect treatment
- 2 days x 0.52 mg/day x 11.3 ng Cu/mg = 11.8 ng Cu direct treatment
- 2 days x 0.46 mg/day x 36.1 ng Cu/mg = 33.2 ng Cu combined treatment

When correcting for this potential but substantially overestimated and unlikely bias, the indirect effect path still results in a significantly increased Cu content (Table S2) at a comparable effect size, which was 27% if the Cu concentration in gammarids was corrected for the Cu gut content (Fig. S1) and 33% if it was not corrected for this bias.

Table S2. ANOVA-table for corrected Cu body burdens of *Gammarus*. All *P*-values <0.05 are printed in bold.

Source of variation	d.f.	SS	MS	*F*	*P*
Indirect	1	47.2	47.2	7.5	**0.026**
Direct	1	897.9	897.9	142.6	**<0.001**
Indirect x Direct	1	371.9	371.9	59.1	**<0.001**
Residuals	8	50.4	6.3		

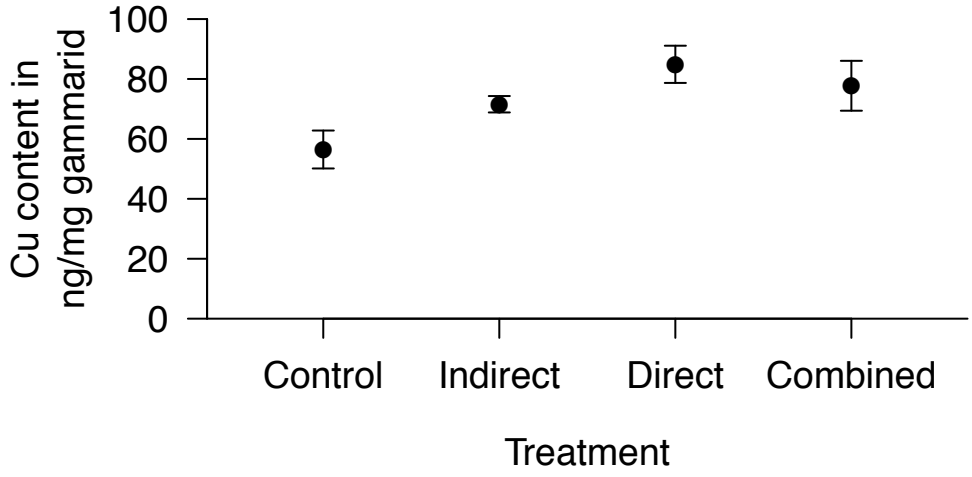

Fig. S1. Means with 95% CIs of corrected Cu content (*n*=3) of *G. fossarum* being subjected for 24 days to four treatments: a Cu free control, gammarids receiving leaves conditioned in the presence of Cu (i.e., indirect), gammarids being directly exposed to Cu (i.e., direct), and a combination of the indirect and direct treatments. For statistical evaluation see Table S2.

LITERATURE CITED IN THIS SUPPLEMENT

Willoughby, L. G., Earnshaw, R., 1982. Gut passage times in *Gammarus pulex* (Crustacea, Amphipoda) and aspects of summer feeding in a stony stream. Hydrobiologia 97, 105-117.

APPENDIX A.5

Waterborne toxicity and diet-related effects of fungicides in the key leaf shredder *Gammarus fossarum* (Crustacea: Amphipoda)

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ABSTRACT

Animals involved in leaf litter breakdown (i.e., shredders) play a central role in detritus-based stream food webs, while their fitness and functioning can be impaired by anthropogenic stressors. Particularly fungicides can affect shredders via both waterborne exposure and their diet, namely due to co-ingestion of adsorbed fungicides and shifts in the leaf-associated fungal community, on which shredders' nutrition heavily relies. To understand the relevance of these effect pathways, we used a full 2x2-factorial test design: the leaf material serving as food was microbially colonized for 12 days either in a fungicide-free control or exposed to a mixture of five current-use fungicides (sum concentration of 62.5 µg/L). Similarly, the amphipod shredder *Gammarus fossarum* was subjected to the same treatments but for 24 days. Waterborne exposure reduced leaf consumption by ~20%, which did not fully explain the reduction in feces production (~30%), indicating an enhanced utilization of food to compensate for detoxification mechanisms. This may also explain the reduced feces production (~10%) of gammarids feeding on fungicide-exposed leaves. The reduction may, however, also be caused by a decreased nutritious quality of the leaves indicated by a reduced species richness (~40%) of leaf-associated fungi. However, compensation for these effects by *Gammarus* was seemingly incomplete, since both waterborne exposure and the consumption of the fungicide-affected diet drastically reduced gammarid growth (~110% and ~40%, respectively). Our results thus indicate that fungicide mixtures have the potential for detrimental implications in aquatic ecosystem functioning by affecting shredders via both effect pathways.

KEYWORDS

agriculture – aquatic hyphomycetes – dietary exposure – environmental risk assessment – fungicide mixture – leaf litter breakdown

INTRODUCTION

Energy budgets of stream ecosystems with forested catchments rely heavily on subsidies from riparian vegetation (Fisher and Likens, 1973). The breakdown of allochthonous leaf litter thus is a critical ecosystem level process (Gessner et al., 1999) being mainly mediated by decomposing microorganisms and detritivorous, leaf-shredding macroinvertebrates (Cummins and Klug, 1979). Although microbial

decomposers contribute relatively little to the leaf mass loss (Hieber and Gessner, 2002), particularly fungi increase the leaves' nutrient content (e.g., proteins and lipids) and degrade recalcitrant structural leaf components. These transformations make leaves a palatable and nutritious food source for leaf-shredding macroinvertebrates (i.e., microbial conditioning; Bärlocher, 1985). Shredders feeding on the conditioned leaf material represent in turn a key link in detrital food webs by producing finer detritus particles, used as food by collectors (Bundschuh and McKie, in press), and by being an important food source for many predators (MacNeil et al., 1999).

However, aquatic shredder communities and the pivotal functions they provide can be affected by anthropogenic activities such as agriculture and the resulting contamination with pesticides (e.g., Piscart et al., 2009). In this context, fungicides – and antimicrobial substances in general – play a special role: first, many antifungal substances act on biological processes that are highly conserved, are thus not specific to fungi (Stenersen, 2004), and can impact a range of different taxonomic groups of aquatic organisms (Maltby et al., 2009). In consequence, shredders can suffer from toxic effects when subjected to waterborne exposure towards these substances (e.g., Zubrod et al., 2014). Second, fungicides can accumulate on leaf material (Dimitrov et al., 2014; Zubrod et al., 2015a) and may thus cause toxic effects when co-ingested together with the leaf substrate as already reported for other pesticides (e.g., Bundschuh et al., 2013). On the other hand, fungicides can negatively affect fungal leaf decomposers indicating detrimental effects on microbial conditioning and thus food quality of leaf material for shredders (Artigas et al., 2012; Bundschuh et al., 2011; Dimitrov et al., 2014; Flores et al., 2014; Zubrod et al., 2011, 2015a).

However, information about the relative importance of both the waterborne and diet-related pathways for shredders is scarce: In a previous study, we demonstrated a reduced growth and lipid content of the amphipod shredder *Gammarus fossarum* when fed leaves microbially conditioned in the presence of the fungicide tebuconazole and being exposed to the same fungicide via the water phase. As the applied fungicide concentration (65 µg/L) resulted in a deteriorated leaf-associated fungal community (Zubrod et al., 2011), while being unlikely to trigger any waterborne toxicity (Zubrod et al., 2010), this study provided indirect evidence for the effects being mediated via the diet-related effect pathway. Similarly, the shredder

Table 1. Origin, ecotoxicologically relevant information, and tested concentrations of the assessed fungicides.

Fungicide	Chemical family	Product applied	Supplier	Mode of action [a]	7-day EC_{50} for *G. fossarum*'s feeding activity with 95% confidence intervals (CIs) [b]	Maximum surface water concentration (µg/L)	Tested concentration (µg/L)
Azoxystrobin	Strobilurins	Ortiva	Syngenta Agro GmbH	Inhibition of mitochondrial respiration	90.8 (79.1-102.5)	29.7 [c]	15.0
Carbendazim	Benzimidazoles	Derosal	Bayer CropScience	Inhibition of mitosis and cell division	75.0 (47.5-102.6)	2.5 [d]	7.5
Cyprodinil	Anilino-pyrimidines	Chorus	Syngenta Agro GmbH	Inhibition of amino acid and protein synthesis	50.5 (0.0-103.3)	2.2 [e]	7.5
Quinoxyfen	Quinolines	Fortress 250	Dow AgroSciences GmbH	Perturbation of signal transduction	23.8 (11.5-36.1)	0.02 [d]	7.5
Tebuconazole	Triazoles	Folicur	Bayer CropScience	Inhibition of sterol biosynthesis	197.8 (144.1-251.6)	9.11 [c]	25.0

[a] Fungicide Resistance Action Committee (2015)

[b] Zubrod et al. (2014)

[c] Berenzen et al. (2005)

[d] Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht Rheinland-Pfalz (2011)

[e] Süss et al. (2006)

Echinogammarus berilloni had a lower leaf consumption and fitness induced by the fungicide imazalil (100 µg/L) only if waterborne and diet-related pathways acted in combination (Flores et al., 2014). As the latter observations were, however, reported at a fungicide concentration causing substantial mortality in the shredder, the observed sublethal effects likely induced via the diet-related pathway seem comparably unimportant.

To develop a more mechanistic understanding of the interplay between the two effect pathways regarding their sublethal effects on shredders, we employed a full 2x2-factorial test design. The first factor was the absence or presence of a fungicide mixture during microbial conditioning of leaves (12 days), which were fed to our model shredder (i.e., diet-related pathway; a distinction between dietary uptake of fungicides and a change in food quality due to fungicide-induced shifts in the fungal community was beyond the scope of this study). The second factor was the absence or presence of the same fungicide mixture in the medium used for culturing the shredders during a 24-days lasting bioassay (i.e., waterborne pathway). As model shredder we chose the amphipod *G. fossarum*, a key species in many European streams (Dangles et al., 2004). As the detection of fungicide mixtures is very common in the field (Battaglin et al., 2010; Bereswill et al., 2012; Reilly et al., 2012), a mixture composed of five current-use substances with differing modes of toxic action at a sum concentration of 62.5 µg/L (Table 1) was applied. The selection of both the model shredder and the fungicide mixture was prompted by the existence of well-established effect thresholds in terms of fungicide-induced feeding preferences and waterborne toxicity for this combination (Zubrod et al., 2014, 2015a). We hypothesized the fungicide mixture would affect gammarids' energy processing and physiological fitness (judged by growth) via both effect pathways. Moreover, we expected additive action of both paths for all endpoints as recently observed during a comparable experiment using copper as stressor (Zubrod et al., 2015b).

MATERIALS AND METHODS

SOURCES OF LEAVES, MICROORGANISMS, AND GAMMARIDS

Leaves of black alder (*Alnus glutinosa*) were collected in autumn 2012 near Landau, Germany (49°11'N; 8°05'E) and stored at -20°C until further use. As per Zubrod et al. (2011), leaves were deployed for 14 days in fine-mesh bags in the Rodenbach, Germany (49°33'N, 8°02'E), upstream of any agricultural activity, settlement, and

wastewater inlet, to establish a natural microbial community. Back in the laboratory, unconditioned frozen leaves were added to the retrieved leaf material and the mixed leaves were kept in aerated conditioning medium (Dang et al., 2005) at 16±1°C in total darkness for at least another 12 days before being used as microbial inoculum.
Upstream of any agricultural activity, settlement, and wastewater inlet, *G. fossarum* – cryptic lineage B (Feckler et al., 2014) – were kick-sampled in the Hainbach, Germany (49°14'N; 8°03'E), seven days prior to their use in the bioassay. Only adult males (6 to 8 mm body length) being visually non-parasitized were used. Animals were kept in a temperature-controlled chamber at 16±1°C in total darkness, continuously aerated, and fed *ad libitum* with conditioned black alder leaves, while they were adapted to the nutrient medium used during the bioassay (i.e., SAM-5S; Borgmann, 1996) by gradually increasing the ratio of bioassay medium to stream water every other day.

EXPERIMENTAL DESIGN

The experimental design, which is described in more detail in Zubrod et al. (2015b), employed a 2x2 factorial design resulting in four treatments: (i) a control where microbial leaf conditioning and culturing of gammarids took place in fungicide-free medium, (ii) leaf material exposed to fungicides during conditioning, gammarids not (i.e., indirect effect pathway), (iii) gammarids exposed to fungicides, leaf material not (i.e., direct effect pathway), and (iv) leaf material and gammarids exposed to fungicides (i.e., combined effect pathway). All fungicide containing media were dosed with a fungicide mixture composed of five substances with different toxic modes of action (Table 1). The ratio of the single substances in the mixture corresponded roughly to the substances' 7-days EC_{50} for *G. fossarum*'s feeding activity (Table 1), which ensured that the contributions of each fungicide to the waterborne toxicity towards *Gammarus* were similar. A nominal sum concentration of 62.5 µg/L was applied, which was expected to reduce gammarids' feeding by approximately 20% according to Zubrod et al. (2014) and to negatively affect the species richness of leaf-associated fungi following conditioning (Zubrod et al., 2015a) but is unlikely to occur in the field over longer time periods.
To condition leaf material, leaf strips of approximately 4 cm x 7 cm were cut from unconditioned black alder leaves and placed in aerated circular aquaria (*n*=3; 150 strips per aquarium) filled with 14 L of conditioning medium together with 50 g (fresh

weight) of the microbial inoculum. During conditioning (16±1°C; total darkness), medium (with the appropriate fungicide concentrations) was renewed every 3 days to ensure a continuous fungicide exposure (cf. Zubrod et al., 2014). After 12 days, two leaf discs with a diameter of 2 cm were cut from each strip, one disc from each side of the leaf's main vein. Discs were immediately introduced into the bioassay or directed to the analyses of the leaf-associated fungal communities. To provide the gammarids during the bioassay with food of a constant quality, four independent leaf conditionings were performed, each one starting 12 days before the respective leaf discs were introduced into the bioassay.

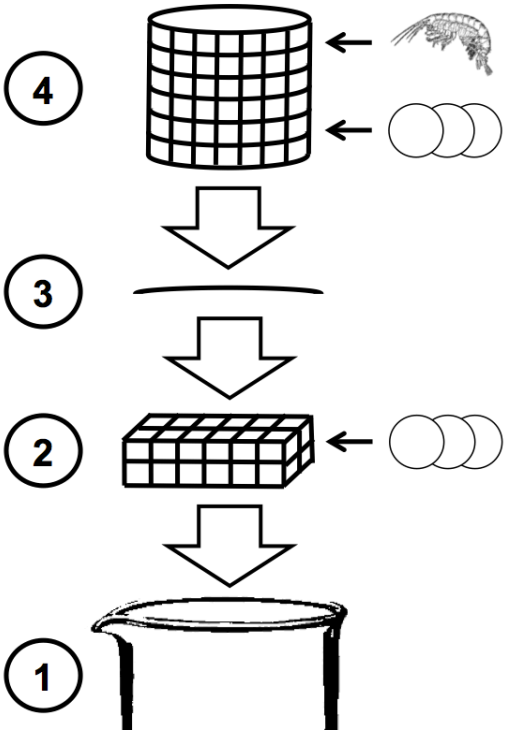

Fig. 1. Scheme illustrating the setup of a bioassay replicate. At the bottom of a (1) 250-mL glass beaker filled with 200 mL bioassay medium a (2) rectangular cuboid cage containing three leaf discs is situated. This cage is topped by a (3) watch glass on which a (4) cylindrical cage containing the corresponding three leaf discs and one gammarid is placed. For more details see text. Reprinted from Zubrod et al. (2015b) with kind permission of Elsevier.

Each replicate of the *Gammarus* bioassay (*n*=65) consisted of a 250-mL glass beaker filled with 200 mL bioassay medium (item 1 in Fig. 1) that was continuously aerated and situated in a temperature-controlled chamber at 16±1°C in total darkness. The beakers were equipped with cylindrical cages made from stainless steel mesh screen (mesh size: 0.5 mm) containing one gammarid together with three leaf discs originating from three separate leaf strips (item 4 in Fig. 1). These cages

facilitated a careful transfer of the animals to new vessels containing fresh bioassay medium (with the appropriate fungicide concentrations) every 3 days (ensuring a continuous exposure; cf. Zubrod et al., 2014) and prevented the animals from coprophagy. In addition, below the cylindrical cage, each beaker contained a rectangular cuboid cage made from the same stainless steel mesh screen (item 2 in Fig. 1). The rectangular cuboid cage contained the corresponding three leaf discs originating from the same three leaf strips as the leaf discs offered as food to the gammarid and allowed to control for handling-related and microbial leaf mass loss. To prevent the feces of the animal from interacting with the leaf discs within the rectangular cuboid cage, a watch glass separated the two cages (item 3 in Fig. 1). Every 6 days (i.e., during every second renewal of the bioassay medium) also the leaf material was replaced, while the leaf disc remains from both cages were dried separately at 60°C for 24 h and weighed to the nearest 0.01 mg to quantify the leaf consumption by *Gammarus*. Moreover, to assess feces production of *Gammarus*, old bioassay medium was filtered through pre-weighed glass fiber filters (GF/6, Whatman, Dassel, Germany). Before the filters were dried and weighed as described above, they were carefully checked to feature exclusively fecal pellets. Any leaf fragments were removed from the filters and added to the recovered leaf disc remains of the respective replicate (see above) to allow an accurate quantification of leaf consumption and feces production. To control for increases in filter weight due to handling and microbial decomposition of leaf material, three additional replicates per treatment were set up without *Gammarus* and treated as the remaining replicates. Mortality of the animals was checked during each medium renewal but was – irrespective of the treatment – negligible (maximum of 3% after 24 days). At the termination of the bioassay, surviving gammarids were shock-frozen in liquid nitrogen, freeze-dried, and weighed to the nearest 0.01 mg.

LEAF-ASSOCIATED FUNGAL COMMUNITIES

At the time food was replaced in the bioassay, samples for characterizing the leaf-associated fungal communities were taken (n=12; three replicate aquaria x four conditioning runs). Five leaf strips arising from one conditioning aquarium were combined to a replicate and analyzed for their ergosterol content as a proxy for fungal biomass according to Gessner (2005): After extraction in alkaline methanol and purification by solid-phase extraction (Sep-Pak® Vac RC tC18 500 mg sorbent,

Waters, Milford, MA, USA), ergosterol was quantified by high-performance liquid chromatography (1200 Series, Agilent Technologies, Santa Clara, CA, USA) using a LiChrospher® 100 RP 18-5 m column (250.0 mm x 4.6 mm, particle size 5 mm, CS-Chromatographie Service, Langerwehe, Germany) at a wavelength of 282 nm.

Fungal species richness was assessed via spore morphology (note a potential bias compared to molecular approaches; e.g., Fernandes et al., in press) following generally the methodology described for instance by Pascoal and Cássio (2004). Three leaf discs (diameter = 16 mm) per replicate aquarium were placed in Erlenmeyer flasks containing 20 mL of deionized water. The Erlenmeyer flasks were placed on an orbital shaker set at 75 rpm in a temperature-controlled chamber at 16±1°C in total darkness for 96 h. After incubation, Tween 80 was added to prevent fungal spores to attach to the leaf discs and facilitate a homogenous distribution of fungal spores. The resulting suspension was filtered onto a membrane filter (HAWG047S6, pore size 0.45 µm, diameter 47 mm, Merck Millipore, Darmstadt, Germany). Half of the filter was then mounted on a microscope slide and spores were fixed and stained by adding lactophenol cotton blue. Stained spores were identified and counted using a microscope (100-fold magnification, BA 300, Motic, Wetzlar, Germany) and several identification keys (mainly Ingold, 1975) until 200 mm^2 of the filters were scanned or a total of 300 spores was counted.

FUNGICIDE ANALYSIS

Fungicide concentrations were measured in fresh and 3-days-old (i.e., before medium renewal) conditioning and bioassay medium (*n*=4) using an ultra-high-performance liquid chromatography (UHPLC-MS) system (Thermo Fisher Scientific, Bremen, Germany; unless specified otherwise) consisting of a Combi PAL autosampler (CTC Analytics, Zwingen, Schweiz) as well as an Accela pump, a Hypersil GOLD C18 column (50.0 mm x 2.1 mm, particle size 1.9 µm), and an Exactive system. Eluent A was Milli-Q water (Merck Millipore, Billerica, MA, USA) and eluent B was methanol (LC-MS grade). Both eluents contained 4.0 mM ammonium formate and 0.1% formic acid. Subsamples of 20 µL were injected to the column with 95% A and 5% B at a flow rate of 0.2 mL/min. Starting 2 min after injection, the analytical column was run isocratically for 6 min at 0% A and 100% B with a flow rate of 0.2 mL/min before rinsing with 5% A and 95% B for 2 min. Flow rates of sheath and auxiliary gas (both nitrogen) were 30 mL/min and 5 mL/min,

respectively. Fungicides were ionized using an Ion Max API source with an ESI probe operated with a spray voltage of 3.5 kV and were quantified in the positive mode at the accurate masses of m/z 404.124, 192.0767, 226.1339, 308.0039, and 308.1522 for azoxystrobin, carbendazim, cyprodinil, quinoxyfen, and tebuconazole, respectively. External calibrations with either conditioning or bioassay medium were used.

Nominal and measured initial fungicide concentrations were in good agreement with a mean deviation of 8%, indicating an accurate dosage of the fungicides and justifying the use of nominal concentrations throughout this paper (Table 2). Moreover, in the treatment assessing the indirect effect pathway, the presence of fungicide-exposed leaf discs did either not result in fungicide water concentrations above the limits of detection or – as for tebuconazole – only up to levels that were more than 40 times lower than reported ecotoxicological thresholds for waterborne toxicity, i.e., 1.9 µg/L (Table 2) versus a 7-day EC_{20} for feeding of 91.2 µg/L (Zubrod et al., 2014). This justifies that effects in this treatment were the result of consumption of the provided leaf material and not of toxicity arising from desorption of fungicides to the water phase.

CALCULATIONS AND STATISTICS

Gammarids' leaf consumption (*C*) is expressed as mg consumed leaf dry mass per gammarid and day and is given by (Zubrod et al., 2011)

$$C = \frac{L_n - L_f}{t}, \qquad (1)$$

where L_f represents the dry weight of the leaf discs available to the gammarids, L_n is the dry weight of the leaf discs prevented from feeding in the rectangular cuboid cages (Fig. 1), and *t* is the observation time, i.e., 6 days. Feces production (*F*) expressed as mg dry mass of feces per gammarid and day was calculated as per Zubrod et al. (2011) as

$$F = \frac{F_a - F_b - F_n}{t}, \qquad (2)$$

where F_a and F_b represent the final and initial dry weight of the filter, respectively, and F_n is the mean increase in dry weight of the filters correcting for filter weight increases due to handling and microbial decomposition of leaf material. For leaf consumption and feces production, observations made on each animal were averaged to display the treatments' effects over the whole experimental duration.

Table 2. Nominal and measured (means with 95% CIs; *n*=4) fungicide concentrations in fresh and 3-days-old conditioning and bioassay medium. The limits of detection (LODs) for conditioning and bioassay medium, respectively, were 0.1 and 0.1 for azoxystrobin, 0.2 and 0.4 for carbendazim, 0.2 and 0.7 for cyprodinil, 0.1 and 0.4 for quinoxfen, and 0.2 and 0.4 for tebuconazole. The limits of quantification (LOQs) for conditioning and bioassay medium, respectively, were 0.2 and 0.3 for azoxystrobin, 0.4 for 1.1 for carbendazim, 0.6 and 1.9 for cyprodinil, 0.3 and 1.1 for quinoxfen, and 0.6 and 1.2 for tebuconazole.

Medium	Treatment	Fungicide	Nominal concentration	Fresh medium	3-days-old
Conditioning	Control	Azoxystrobin	0	<LOD	<LOD
		Carbendazim	0	<LOD	<LOD
		Cyprodinil	0	<LOD	<LOD
		Quinoxyfen	0	<LOD	<LOD
		Tebuconazole	0	<LOD	<LOD
	Fungicide exposure	Azoxystrobin	15	13.7 (11.5-16.0)	2.3 (0.7-3.9)
		Carbendazim	7.5	7.3 (6.7-8.0)	6.6 (5.0-8.3)
		Cyprodinil	7.5	6.2 (4.5-7.8)	<LOQ
		Quinoxyfen	7.5	7.8 (6.6-8.9)	<LOQ
		Tebuconazole	25	26.2 (20.3-32.0)	6.6 (3.8-9.3)
Bioassay	Control	Azoxystrobin	0	<LOD[a]	<LOD
		Carbendazim	0	<LOD[a]	<LOD
		Cyprodinil	0	<LOD[a]	<LOD
		Quinoxyfen	0	<LOD[a]	<LOD
		Tebuconazole	0	<LOD[a]	<LOD
	Indirect	Azoxystrobin	0	<LOD[a]	<LOD
		Carbendazim	0	<LOD[a]	<LOD
		Cyprodinil	0	<LOD[a]	<LOD
		Quinoxyfen	0	<LOD[a]	<LOD
		Tebuconazole	0	<LOD[a]	1.9 (1.2-2.5)
	Direct	Azoxystrobin	15	13.3 (12.0-14.6)[b]	11.6 (10.4-12.8)
		Carbendazim	7.5	7.6 (4.8-10.4)[b]	8.3 (7.5-9.1)
		Cyprodinil	7.5	6.5 (5.1-7.8)[b]	3.7 (3.0-4.5)
		Quinoxyfen	7.5	7.2 (5.8-8.5)[b]	1.2 (0.3-2.1)
		Tebuconazole	25	26.9 (19.2-34.5)[b]	23.0 (19.3-26.6)
	Combined	Azoxystrobin	15	13.3 (12.0-14.6)[b]	12.2 (11.1-13.2)
		Carbendazim	7.5	7.6 (4.8-10.4)[b]	10.1 (7.4-12.9)
		Cyprodinil	7.5	6.5 (5.1-7.8)[b]	4.2 (3.8-4.7)
		Quinoxyfen	7.5	7.2 (5.8-8.5)[b]	1.3 (0.15-2.5)
		Tebuconazole	25	26.9 (19.2-34.5)[b]	24.0 (19.7-28.2)

[a] & [b] Concentrations with the same letter were determined in the same four replicates

Animals' growth (in terms of dry weight gain) during the bioassay was determined by subtracting the mean dry weight of 130 gammarids at the start of the bioassay (using one measurement) from the final dry weight of each animal used in the bioassay.

Data were visually checked for normality and Levene's test was used to assess homoscedasticity. For all gammarid-related endpoints, two-way ANOVAs were either performed on the original data (growth) or, if the presumptions for parametric testing were violated, on ranks (leaf consumption and feces production). Fungal biomass associated with the leaf material was assessed using Student's *t*-test, while for fungal species richness – due to non-normality – the Wilcoxon rank-sum test was used. R version 3.2.0 for Mac (R Development Core Team, 2015) was used for all statistics and figures.

RESULTS AND DISCUSSION

WATERBORNE AND DIET-RELATED EFFECTS IN *GAMMARUS*

Waterborne (direct) exposure to the fungicide mixture resulted in a significant impairment in the leaf consumption of *Gammarus* by approximately 20% (Fig. 2; Table 3). Accordingly, also the feces production of animals subjected to this pathway was significantly reduced (~30%; Fig. 2; Table 3). However, the reduction in feeding did not fully account for the impairment in feces production, which is emphasized by the still significant decrease in the latter when expressed relative to leaf consumption (~15%; Fig. 2; Table 3). This stronger reduction in egestion relative to ingestion may point to an enhanced utilization of food as a compensational mechanism to cover the increased energy demand due to toxic stress (Maltby, 1999).

The diet-related effect pathway (i.e., dietary uptake and/or fungicide-mediated shifts in food quality), in contrast, did not result in implications in leaf consumption but in a significant decrease in gammarids' feces production (~10%; Fig. 2; Table 3). The latter may be related to compensation of toxic effects induced due to the co-ingestion of fungicides adsorbed to the leaf substrate (cf. Zubrod et al., 2015a). In addition, an altered food quality potentially triggered by shifts in the fungal community associated with the leaf material conditioned in presence of the fungicide mixture may have triggered this diet-related effect. Although total fungal biomass of the leaf material was not significantly affected by fungicide exposure (P = 0.715; Fig. 3a), fungal species richness (lying in the range of published values for the control; e.g., Zubrod et al., 2015a), which was considered to trigger food quality for *Gammarus* (Bundschuh et al., 2011), was statistically significantly reduced (~40%; P = 0.041; note also the fungicide-induced reduction in variability associated with this endpoint; Fig. 3b). Moreover and as observed before (cf. Bundschuh et al., 2011; Zubrod et al.,

2015a), fungicide exposure increased the contribution of *Heliscus lugdunensis* SACCARDO & THERRY and *Tetracladium marchalianum* DE WILDEMAN, two species rejected by gammarids (Arsuffi and Suberkropp, 1989; Gonçalves et al., 2014), to total fungal sporulation (see Supplementary Material). Presuming that an active rejection of a fungal species indicates a lower food quality for a shredder, the observed fungicide-induced changes in the leaf-associated fungal community point to a lower food quality for *Gammarus* relative to leaf material conditioned under control conditions.

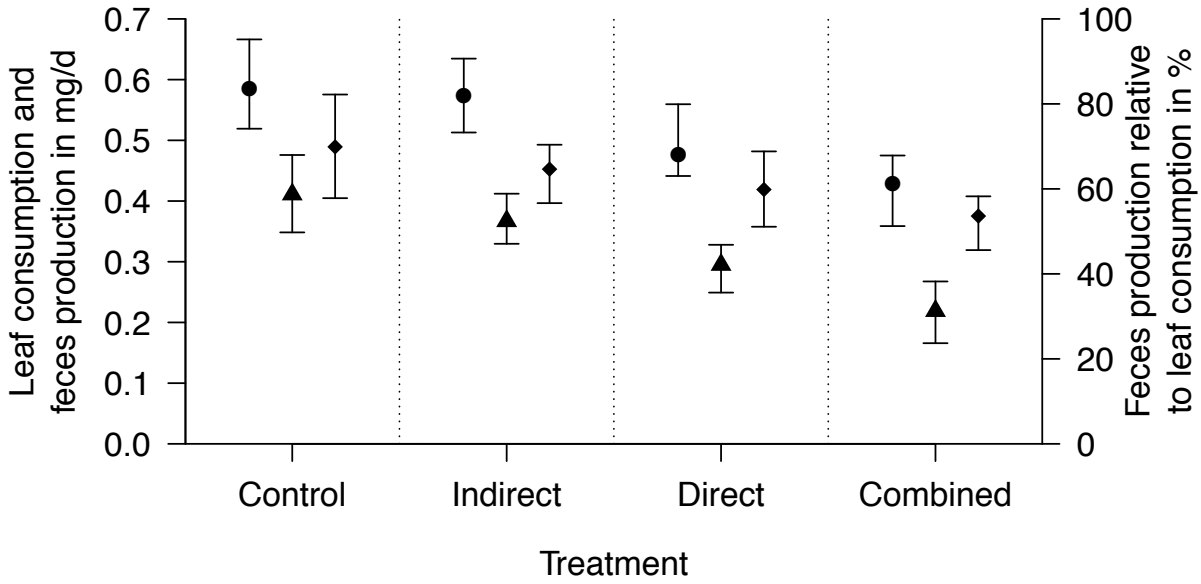

Fig. 2. Medians with 95% CIs of leaf consumption (points) and absolute (triangles) and relative (diamonds) feces production of *G. fossarum* being subjected for 24 days to four treatments: a fungicide-free control, gammarids receiving leaves conditioned in the presence of fungicides (i.e., indirect), gammarids being directly exposed to fungicides (i.e., direct), and a combination of the indirect and direct treatments. For statistical evaluation see Table 3.

The consumption of such leaves conditioned in presence of the fungicide mixture over 24 days resulted in a significantly reduced gammarid growth (~40%), as was also caused by waterborne exposure (~110%; Fig. 4; Table 3). These data suggest that the enhanced utilization of the ingested food indicated by the reduction in feces production (Fig. 2) did not fully compensate for the additional energy needed to cope with the stress triggered via waterborne or diet-related pathways (Maltby, 1999). The resulting energy budget did not support a significant growth for animals subjected to either pathway (zero included in 95% confidence intervals; Fig. 4), while both pathways acted additively (no significant interaction of pathways; Table 3) and thus resulted in a significant loss of weight over the experimental period when combined

(Fig. 4). Similarly, additive action (non-significant interaction terms; Table 3) of the two effect paths was apparent for the endpoints related to the energy processing of *Gammarus*: substantial reductions in leaf consumption and feces production by ~30% and ~50%, respectively, were observed when both pathways were combined (Fig. 2), which represents the realistic worst-case situation for shredders inhabiting fungicide-affected streams (cf. Zubrod et al., 2011).

Table 3. ANOVA-tables for all gammarid-related endpoints. All *P*-values <0.05 are printed in bold.

Endpoint	Source	d.f.	SS	MS	*F*-value	*P*-value
Leaf consumption	Food	1	10,122	10,122	2.17	0.142
	Water	1	97,946	97,946	21.02	**<0.001**
	Food x Water	1	4,673	4,673	1.00	0.318
	Residuals	242	1,127,811	4,660		
Feces production (absolute)	Food	1	53,771	53,771	13.80	**<0.001**
	Water	1	237,264	237,264	60.88	**<0.001**
	Food x Water	1	6,301	6,301	1.62	0.205
	Residuals	242	943,207	3,898		
Feces production (relative)	Food	1	31,938	31,938	6.70	**0.010**
	Water	1	51,994	51,994	10.91	**0.001**
	Food x Water	1	2,962	2,962	0.62	0.431
	Residuals	242	1,153,663	4,767		
Growth	Food	1	3.54	3.54	4.63	**0.033**
	Water	1	11.62	11.62	15.21	**<0.001**
	Food x Water	1	0.95	0.95	1.24	0.266
	Residuals	241	184.18	0.76		

Source: Source of variation, i.e., the single factors (exposure to the diet-related (=Food) and waterborne (=Water) effect pathway), their interaction, and the model residuals

d.f.: Degrees of freedom

SS: Sums of squares

MS: Mean squares

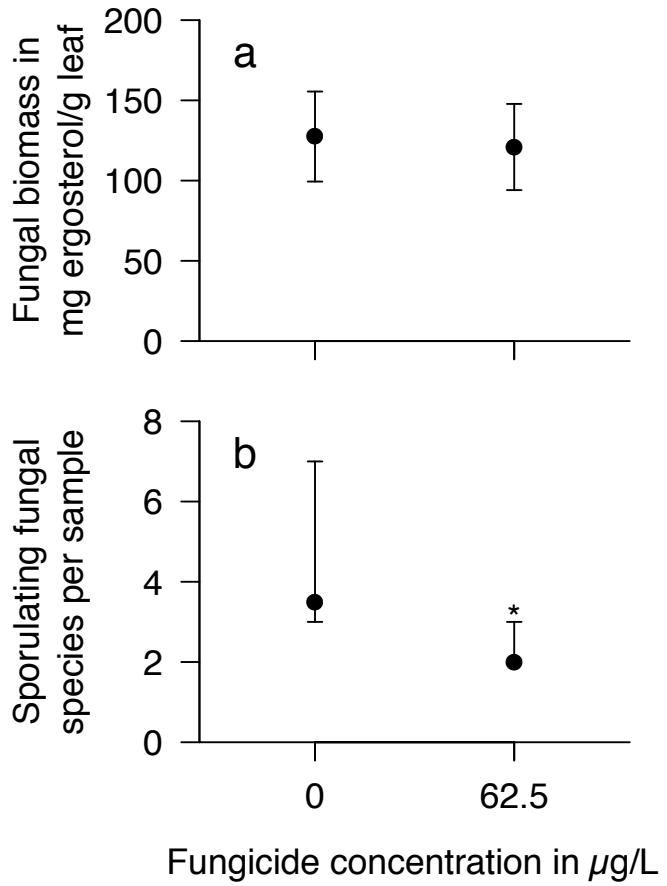

Fig. 3. Mean fungal biomass (a) and median species richness of sporulating fungal taxa (b) associated with leaf material – with 95% CIs – exposed to fungicide sum concentrations of 0 (i.e., control) or 62.5 µg/L during microbial conditioning. The asterisk denotes a statistically significant difference.

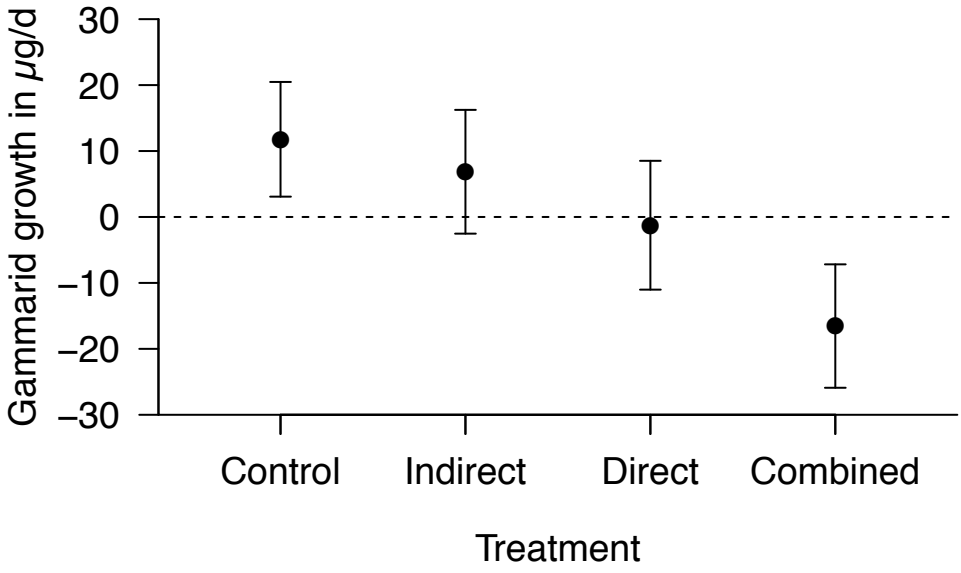

Fig. 4. Mean growth of *G. fossarum* – with 95% CIs – being subjected for 24 days to four treatments: a fungicide-free control, gammarids receiving leaves conditioned in the presence of fungicides (i.e., indirect), gammarids being directly exposed to fungicides (i.e., direct), and a combination of the indirect and direct treatments. For statistical evaluation see Table 3.

POTENTIAL IMPLICATIONS FOR DETRITUS-BASED FOOD WEBS

Although the sum fungicide concentration applied may not be measured in surface waters over 12 and 24 days, respectively, our results still indicate that fungicide mixtures – and antimicrobial substances in general – have the potential to cause detrimental effects in detritus-based stream food webs by affecting shredders via both waterborne exposure and diet-related exposure and effects over the long term. Maltby et al. (2002), for instance, showed that gammarids' leaf consumption is correlated with in-stream leaf litter breakdown. As effects on leaf consumption may thus directly translate into less leaf-bound energy becoming available to the stream food web, reductions in this endpoint as observed for animals subjected to waterborne or combined exposure (Fig. 2) appear to be of high ecological significance. Furthermore, since feces is considered a high quality food resource in stream food webs (Bundschuh and McKie, in press), the observed reduction in feces production (Fig. 2) triggered by both pathways may impact the food availability for collecting species but also juvenile gammarids that feed on the adult animals' feces (McCahon and Pascoe, 1988).

Moreover, animals are only able to grow if energy is available in excess to the requirements for maintenance (Sibly and Calow, 1986). Both waterborne exposure and a fungicide-affected diet reduced this margin (Fig. 4). Finally, considering that the body size (i.e., the result of growth) and reproductive success are highly correlated in gammarids (Glazier, 2000), an impaired reproductive capacity may be the consequence. This may result in a lower abundance of gammarids further aggravating the potentially impaired in-stream energy transfer along the heterotrophic food web. In addition, gammarids represent a key prey for a variety of aquatic and terrestrial predators (MacNeil et al., 1999). Fungicide-induced impairments in gammarids' performance triggered by waterborne exposure or diet-related exposure and effects (*inter alia* reduced reproduction and body size) may thus ultimately affect higher trophic levels in heterotrophic food webs (Wallace and Eggert, 1997) and even across ecosystem boundaries (Paetzold et al., 2011), which is – due to the lack of data – a matter of debate. All in all, this indicates the need for a thorough empirical assessment of the proposed fungicide-induced impairments in pivotal ecosystem functions provided by shredders via population-level experiments as well as community-level assessments using mesocosm and field studies at realistic exposure regimes.

IMPLICATIONS FOR ENVIRONMENTAL RISK ASSESSMENT

Although our data suggest the effect path related to waterborne exposure to cause stronger effects than dietary uptake of fungicides and fungicide-mediated shifts in food quality (judged by effect sizes, Figs. 2 and 4), the general applicability of this phenomenon may depend on the combination of stressors and concentration. Aquatic hyphomycetes' sensitivity towards most fungicides appears to be higher than that of *Gammarus* (Zubrod et al., 2014; Zubrod et al., 2015a). Consequently, at fungicide concentrations below sublethal effect thresholds for the waterborne effect pathway but still sufficiently high to alter the leaf-associated fungal community and thus the nutritious value of the leaf material for shredders, an opposite pattern may be observed. In the field, the latter scenario seems more likely as the prophylactic use of fungicides (with up to ten applications per season) creates the potential for long-term and/or repeated pulse exposures towards fungicides but at concentrations below those assessed in the present study (e.g., Battaglin et al., 2010; Reilly et al., 2012) reducing the risk for waterborne toxicity at the shredder-level (cf. Fernández et al., 2015; Zubrod et al., 2014). In contrast, detrimental effects on fungal species richness can be observed at fungicide concentrations in the low µg/L-range (Zubrod et al., 2015a), which directs more attention towards the diet-related effect pathway (cf. Pacioglu et al., in press; Zubrod et al., 2015b), particularly since gammarids do not actively avoid fungicide-affected leaf material in the field (Fernández et al., 2015). However, even at fungicide concentrations resulting in sublethal toxicity in shredders via waterborne exposure such as that applied during this study, diet-related effects can contribute considerably to the combined effect of fungicides (Fig. 2 and 4). Ignoring the diet-related pathway may thus underestimate the risk posed by fungicides and a broad range of other classes of chemical stressors such as insecticides and metals (e.g., Bundschuh et al., 2013; Pacioglu et al., in press; Zubrod et al., 2015b).

Hence, the present study strongly points to the knowledge gap related to the role of diet-related effects, which seems not to be adequately covered by environmental risk assessment. To overcome this shortcoming it may be a sensible first step to more systematically assess the adsorption dynamics of fungicides and other chemical stressors (i.e., pesticides and metals; Bundschuh et al., 2013; Zubrod et al., 2015b) onto leaf material and the resulting toxicity triggered by its ingestion. Combining these efforts with a more detailed understanding of the development and composition

of leaf-associated microbial communities by involving for instance molecular biological tools (Fernandes et al., 2011) would help to unravel the significance of both factors likely driving the quality of leaves for shredders. Thereby, reasonable refinements for environmental risk assessment can be developed, ultimately safeguarding the integrity of aquatic ecosystems.

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SUPPLEMENTARY INFORMATION

FURTHER INFORMATION ABOUT THE LEAF-ASSOCIATED FUNGAL COMMUNITIES

In total, the spores of 16 fungal species were identified on leaf material during the present study. The communities were dominated by three species (i.e., *Clavatospora longibrachiata*, *Heliscella stellata*, and *Tetracladium marchalianum*) contributing on average 96% and 82% to total sporulation in the controls and the fungicide treatment, respectively (Table A1). In addition to the reduction in species richness discussed in the main body of the manuscript, fungicide exposure significantly reduced total spore production by more than 90% (Wilcoxon rank sum test; P = 0.004; Fig. A1). Moreover, fungicide-exposed leaves featured a fungal community that was significantly different from the control (Permutational Multivariate Analysis of Variance performed on Bray−Curtis dissimilarities; P = <0.001; Fig. A2). The results of a similarity percentage analysis (SIMPER) indicate that the three dominant species accounted for most (85%) of these dissimilarities (Table A1).

Table A1. List of all fungal species identified in the control and fungicide treatment during the present study with their mean contributions (in %) to total sporulation and associated standard errors. Species in brackets were identified in only one sample of the experiment. Additionally, the percentage contribution to community differences (based on SIMPER analysis) are provided.

Species	Control	Fungicide treatment	Percentage contribution (SIMPER)
[*Alatospora acuminata* Ingold]	<1%	<1%	0.4
Anguillospora crassa Ingold	<1%	<1%	1.3
[*Anguillospora longissima* (Saccardo & P. Sydow) Ingold]	<1%	<1%	0.2
[*Clavariopsis aquatica* De Wildeman]	<1%	<1%	0.3
Clavatospora longibrachiata (Ingold) Sv. Nilsson ex Marvanová & Sv. Nilsson	46.1±7.6	51.6±11.1	34.8
Dactylella submersa (Ingold) Sv. Nilsson	2.4±1.9	2.8±2.8	4.1
[*Flagellospora penicillioides* Ingold]	<1%	<1%	0.4
Heliscella stellata (Ingold & V.J. Cox) Marvanová	36.1±8.4	2.3±2.0	32.9
Heliscus lugdunensis Saccardo & Therry	<1%	8.8±6.2	3.0
Lemonniera aquatica De Wildeman	<1%	<1%	2.0
[*Lemonniera terrestris* Tubaki]	<1%	<1%	0.3
[*Sigmoidea aurantiaca* Descals]	<1%	1.9±1.9	0.8
Tetracladium marchalianum De Wildeman	14.0±8.2	27.6±8.5	16.9
[*Tetracladium setigerum* (Grove) Ingold]	<1%	<1%	0.3
Tricladium angulatum Ingold	<1%	<1%	1.1
Unknown hyphomycete	<1%	3.4±2.8	1.1

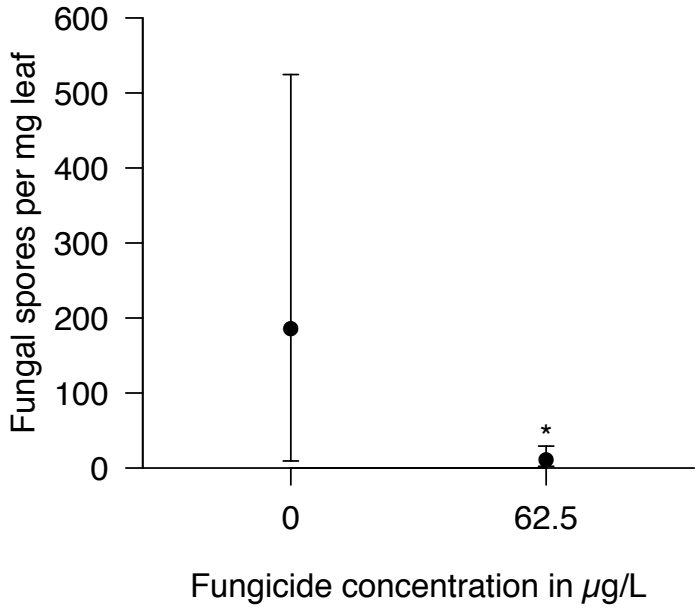

Fig. A1. Median number of fungal spores per mg leaf material – with 95% CIs – exposed to fungicide sum concentrations of 0 (i.e., control) or 62.5 µg/L during microbial conditioning. The asterisk denotes a statistically significant difference.

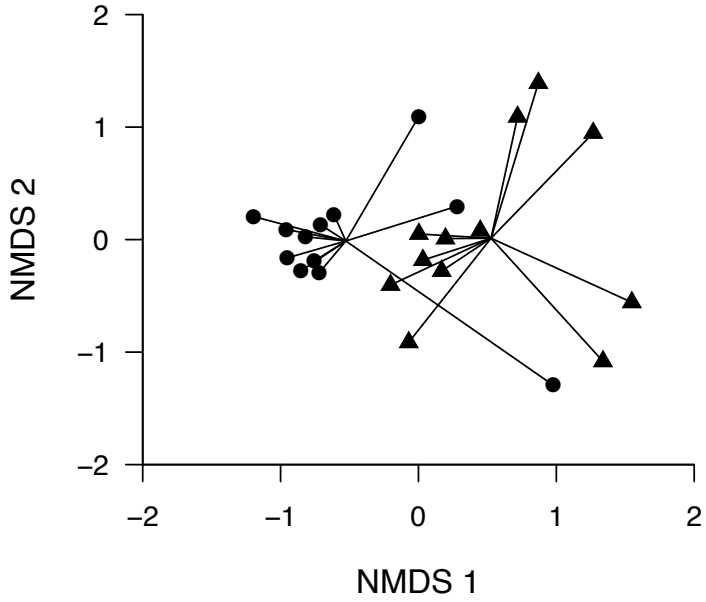

Fig. A2. Non-metric multidimensional scaling (NMDS; an ordination technique to display the dissimilarities of samples; Clarke, 1993) plot of aquatic fungal communities associated with leaf material conditioned in the control (circles) or the fungicide treatment (triangles). A stress value, which provides a measure of "goodness-of-fit" for NMDS (with reasonable fits indicated when below 0.2; Clarke 1993), of 0.113 was determined.

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APPENDIX A.6

CURRICULUM VITAE

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04/2011	M.Sc. Ecotoxicology (thesis title: “Ecotoxicological impact of the fungicide tebuconazole on an aquatic decomposer-detritivore system”)
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2015:

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Zubrod, J. P., Bundschuh, M., Englert, D., Feckler, A., Schulz, R., 2012. (In)Direkte Effekte anthropogener Stressoren auf Gammariden. Colloquium of the department of biogeography. University of Basel, Switzerland. October 8th 2012.

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Newton, K., **Zubrod, J. P.**, Englert, E., Schulz, R., Bundschuh, M., 2015. Systemic fungicides can alter the quality of plant material for aquatic shredders. SETAC Europe 25th Annual Meeting, Barcelona, Spain.

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