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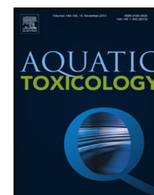

Waterborne toxicity and diet-related effects of fungicides in the key leaf shredder *Gammarus fossarum* (Crustacea: Amphipoda)

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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 2 × 2-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.

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1. 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, 2015), 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 fun-

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gal 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 *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 2 × 2-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).

2. Materials and methods

2.1. 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), 7 days prior to their use in the bioassay. Only adult males (6–8 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.

2.2. Experimental design

The experimental design, which is described in more detail in Zubrod et al. (2015b), employed a 2 × 2-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 × 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.

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

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