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The evil within? Systemic fungicide application in trees enhances litter quality for an aquatic decomposer-detritivore system[☆]

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ABSTRACT

Waterborne exposure towards fungicides is known to trigger negative effects in aquatic leaf-associated microbial decomposers and leaf-shredding macroinvertebrates. We expected similar effects when these organisms use leaf material from terrestrial plants that were treated with systemic fungicides as a food source since the fungicides may remain within the leaves when entering aquatic systems. To test this hypothesis, we treated black alder (*Alnus glutinosa*) trees with a tap water control or a systemic fungicide mixture (azoxystrobin, cyprodinil, quinoxyfen, and tebuconazole) at two worst-case application rates. Leaves of these trees were used in an experiment targeting alterations in two functions provided by leaf-associated microorganisms, namely the decomposition and conditioning of leaf material. The latter was addressed via the food-choice response of the amphipod shredder *Gammarus fossarum*. During a second experiment, the potential impact of long-term consumption of leaves from trees treated with systemic fungicides on *G. fossarum* was assessed. Systemic fungicide treatment altered the resource quality of the leaf material resulting in trends of increased fungal spore production and an altered community composition of leaf-associated fungi. These changes in turn caused a significant preference of *Gammarus* for microbially conditioned leaves that had received the highest fungicide treatment over control leaves. This higher food quality ultimately resulted in a higher gammarid growth (up to 300% increase) during the long-term feeding assay. Although the underlying mechanisms still need to be addressed, the present study demonstrates a positive indirect response in aquatic organisms due to systemic pesticide application in a terrestrial system. As the effects from the introduction of plant material treated with systemic fungicides strongly differ from those mediated via other pathways (e.g., waterborne exposure), our study provides a novel perspective of fungicide-triggered effects in aquatic detritus-based food webs.

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1. Introduction

Leaf litter breakdown is an essential ecosystem level process in streams providing carbon, nutrients and energy to local as well as

downstream food webs (Vannote et al., 1980). The microbial decomposition of leaf litter, considered to be mainly driven by aquatic hyphomycete fungi (Suberkropp and Klug, 1976), contributes considerably to leaf breakdown (Taylor and Chauvet, 2014). Moreover, these fungi increase the nutritional quality of leaf litter for detritivorous macroinvertebrates (i.e., shredders) by breaking down structural polysaccharides and elevating concentrations of proteins and lipids (i.e., conditioning; Bärlocher, 1985). Shredders in turn produce fine particulate organic matter (e.g., feces), which provides food for collectors (Bundschuh and McKie, 2016), and are important prey for many aquatic and terrestrial predators (e.g., MacNeil et al., 1999).

These decomposer-detritivore systems can, however, be

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affected by anthropogenic stressors, such as pesticides, that enter streams via point and non-point sources (e.g., Neumann et al., 2002). Fungicides seem to be of particular concern as both leaf-associated microorganisms and shredders respond to fungicide stress at environmentally relevant concentrations (Baudy et al., 2017; Zubrod et al., 2015a). This high sensitivity of both organism groups can be related to the fungicides' action at highly conserved molecular targets (Stenersen, 2004). Consequently, shredders are potentially affected via two pathways: either through waterborne toxicity (e.g., Baudy et al., 2017), or a reduced nutritional quality of the leaf litter due to fungicide-induced modifications of the leaf-associated microbial community (e.g., Bundschuh et al., 2011). Systemic fungicides, however, may offer an additional, but not yet considered, exposure pathway as they are taken up and translocated within exposed plants (Stenersen, 2004). While crops are intentionally treated with fungicides for protection against fungal pests, fungicides are unintentionally introduced into non-target areas such as field margins during and following their application. For instance, for cereals, Schmitz et al. (2013) calculated an average pesticide input over the first meter of a field margin of approximately 40% of the applied field rate due to spray drift and overspray. As a consequence, tree species harboured by these field margins (cf. Ucar and Hall, 2001) may take up systemic fungicides, which may result in plant material containing these substances to enter adjacent surface waters during leaf fall. Consequently, microorganisms and shredders may be exposed to systemic fungicides via their diet (cf. Englert et al., 2017b), while potential effects have yet to be studied.

To address this knowledge gap, we used leaves from black alder (*Alnus glutinosa* (L.) GAERTN.) trees treated twice with a systemic fungicide mixture. These leaves served as the basis for two experiments. In the first experiment, we focused on potential effects of systemic fungicides on the functions provided by leaf-associated microorganisms, namely the decomposition and conditioning of leaf material. Conditioning was assessed via the food choice of *Gammarus fossarum* KOCH (Crustacea, Amphipoda), a key shredder in low-order streams (e.g., Englert et al., 2013), which shows a highly selective feeding pattern (Arsuffi and Suberkropp, 1989). To gain a mechanistic understanding of potential functional effects, this experiment was supplemented by the quantification of several endpoints related to fungal community performance and structure (i.e., biomass, spore production, and community composition) as well as leaf litter quality (i.e., stoichiometry). In the second experiment, the potential impact of long-term consumption of systemic fungicide-treated, microbially conditioned leaves on leaf processing (i.e., leaf consumption and feces production) and physiology (i.e., growth and lipid content) of *G. fossarum* was examined during a 24-day feeding study. Analogous to waterborne fungicide exposure, we anticipated negative effects on microbial decomposer communities when colonizing systemic fungicide-treated leaves, which would in turn negatively affect leaf palatability for gammarids (Bundschuh et al., 2011). Long-term consumption of such lower-quality food was, furthermore, expected to modify gammarids' energy processing and have negative implications on their physiology (Zubrod et al., 2015b).

2. Materials & methods

2.1. Sources of systemic fungicide-treated leaves and test animals

Ten three to four years old black alder trees, unexposed to pesticides prior to their involvement in this study, were purchased in April 2014 from an ecological tree nursery (Baumschule von der Mühlen, Küsten, Germany). Black alder trees were selected as a model because *Alnus* spp. are key riparian tree species in Europe,

and large parts of North America and Asia (as reviewed by Bjelke et al., 2016). Consequently, they occur in riparian buffer strips that may receive pesticide inputs, for instance, via spray drift (e.g., Ohliger and Schulz, 2010). The trees were placed in 3-L pots and irrigated with tap water three times a day for 10–15 min (Englert et al., 2017a). In mid-July 2014, the trees (mean \pm standard deviation; height: 191.5 ± 9.7 cm; diameter at breast height: 8.9 ± 0.1 mm) were treated with a systemic fungicide mixture at three levels including an uncontaminated control. The four trees serving as the control received 0.5 L tap water. Three additional trees received 0.5 L tap water including azoxystrobin, cyprodinil, quinoxyfen, and tebuconazole at concentrations abstracted from recommended field rates (averaged over a variety of crops; BVL, 2014; Table 1), assuming roots of *A. glutinosa* trees at the assessed size take up water from 1 m^2 of soil. This treatment simulated a worst-case exposure scenario (i.e., direct overspray of riparian zones) and considered the frequent detection of fungicides in mixtures in non-target ecosystems (e.g., Hvezdova et al., 2018; Reilly et al., 2012). Moreover, another three trees received 0.5 L tap water containing ten times the field rates (Table 1). This over-dose treatment was conducted to indicate concentration-dependent effects. After six weeks, all treatments were applied a second time to account for the repetitive application of fungicides during the growing season (Smalling et al., 2013). After another six weeks, in October 2014 and coinciding with the time of abscission, leaves were handpicked and stored at -20°C until use. At that time, trees' mean relative increases in height and diameter at breast height were $22.1 \pm 7.9\%$ and $107.1 \pm 30.2\%$, respectively, compared to the time of first treatment. As we were primarily interested in testing for the potential ecotoxicological effects induced by systemic fungicides contained in leaves, the development of analytical methods to determine internal fungicide concentrations in leaf material (cf. Englert et al., 2017a) was beyond the scope of the present study.

Gammarids were collected in the Hainbach near Frankweiler (Germany; $49^\circ 14' \text{N}$, $8^\circ 03' \text{E}$), upstream from any agricultural activity, settlement, and wastewater inlet, which renders previous exposure to the test substances highly unlikely, 7 days prior to use. Only adult males with diameters ranging from 1.6 to 2.0 mm were used to minimize size- and gender-related variability in response variables (Naylor et al., 1989). Gammarids were acclimatized at $16 \pm 1^\circ \text{C}$ in darkness and adapted to the test medium (i.e., SAM-5S; Borgmann, 1996) by daily increasing the ratio of SAM-5S to stream water. During acclimation, gammarids were fed *ad libitum* with pre-conditioned control black alder leaves. For the food-choice trials (see section 2.2), gammarids were starved for 96 h prior to their use in the experiments to ensure consumption during the trials. At the start of the long-term feeding assay (see section 2.3), 35 gammarids were shock-frozen in liquid nitrogen and stored at -80°C .

2.2. Food-choice trials

Food-choice trials were performed in general as per Bundschuh et al. (2011). To differentiate between systemic fungicide effects on leaf palatability for *G. fossarum* due to fungicide residues in the leaves and fungicide-induced alterations in microorganism-mediated leaf quality, three food-choice trials were performed, two with unconditioned and one with microbially conditioned leaves. For each of the former two, 59 leaf discs for each fungicide treatment and two sets of 59 discs from the systemic fungicide-free control were cut using a 1.6 cm-diameter cork borer. From each set, 49 discs were used in the food-choice trials and 10 discs for the correction of microbial and abiotic leaf mass losses. The leaf discs were freeze-dried for 24 h and weighed to the nearest 0.01 mg. To obtain microbially conditioned leaf material for the third food-choice trial, 49 sets of two leaf discs from 49 individual leaves

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