1 **Exposure pathway-dependent effects of the fungicide epoxiconazole on a**
2 **decomposer-detritivore system**

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Abstract

Shredders play a central role in the breakdown of leaf material in aquatic systems. These organisms and the ecological function they provide may, however, be affected by chemical stressors either as a consequence of direct waterborne exposure or through alterations in food-quality (indirect pathway). To unravel the biological relevance of these effect pathways, we applied a 2x2-factorial test design. Leaf material was microbially colonized for 10 days in absence or presence of the fungicide epoxiconazole (15 µg/L) and subsequently fed to the shredder *Asellus aquaticus* under exposure to epoxiconazole (15 µg/L) or in fungicide-free medium over a 28-day period ($n=40$). Both effect pathways caused alterations in asselids' food processing, physiological fitness, and growth, although not always statistically significantly: assimilation either increased or remained at a similar level relative to the control suggesting compensatory behavior of *A. aquaticus* to cope with the enhanced energy demand for detoxification processes and decreased nutritional quality of the food. The latter was driven by lowered microbial biomasses and the altered composition of fatty acids associated with the leaf material. Even with increased assimilation, direct and indirect effects caused decreases in the growth and lipid (fatty acid) content of *A. aquaticus* with relative effect sizes between 10 and 40%. Moreover, the concentrations of two essential polyunsaturated fatty acids (i.e., arachidonic acid and eicosapentaenoic acid) were non-significantly reduced (up to ~15%) in asselids. This effect was, however, independent of the exposure pathway. Although waterborne effects were generally stronger than the diet-related effects, results suggest impaired functioning of *A. aquaticus* via both effect pathways.

44 **Keywords:** *Asellus aquaticus*; biofilm; fatty acid composition; food quality; leaf litter
45 breakdown; shredder
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1. Introduction

In low order stream ecosystems with forested catchments, detritus from the riparian vegetation (e.g. leaf material) is often the primary source of carbon (C) that fuels aquatic food web productivity (Fisher and Likens, 1973). To incorporate these detrital resources into the food web, the leaf material has to be broken down by microbial decomposers and leaf-shredding macroinvertebrates (i.e., detritivores; Cummins and Klug, 1979). In this process, microbial colonization (i.e., conditioning; in particular by aquatic hyphomycetes) enhances the palatability and nutritional quality of leaf material for detritivores by incorporating nutrients from the water phase and increasing the amount of proteins and lipids such as fatty acids (FA; Arce Funck et al., 2015; Bärlocher, 1985; Danger and Chauvet, 2013). Therefore, feeding on microbially conditioned leaf material allows shredders to maintain survival, growth rate and reproductive output (Graça, 2001).

Anthropogenic stressors like fungicides, however, can affect the ecological integrity of such decomposer-detritivore systems (as reviewed in Feckler et al., 2015). On the one hand, direct effects on shredders have been reported when subjected to fungicides at field-relevant concentrations via the water phase (e.g., Zubrod et al., 2014). Additionally, fungicides may cause toxic effects when sorbed to leaf material (Dimitrov et al., 2014) and are co-ingested during consumption, as already reported for other pesticides (Bundschuh et al., 2013). On the other hand, fungicides may affect shredders indirectly by inhibiting the microbial conditioning process and lowering the palatability of the leaf material (e.g. Zubrod et al., 2015a). Fungicide-induced effects via this indirect pathway, however, have only been studied for two amphipod species within the diverse functional group of shredders, namely *Echinogammarus berilloni* (Flores et al., 2014) and *Gammarus fossarum* (Zubrod et

al., 2011; Zubrod et al., 2015a). Although the abundance of *Gammarus* can be high in undisturbed stream sections, abundance can be relatively low in streams affected by nutrient pollution (e.g., agricultural streams; Whitehurst, 1991). In such impacted streams, the species *Asellus aquaticus* (L.) (Crustacea: Isopoda) often becomes the predominant crustacean shredder (Whitehurst, 1991). *A. aquaticus* thus provides the energy for local and downstream communities by its leaf shredding activity, a function typically attributed to *Gammarus* (Graça et al., 1993). Despite the suggested higher tolerance of *A. aquaticus* towards nutrient stress, its behavior and survival has been shown to be affected by pesticides (e.g., Beketov and Liess, 2008; Bundschuh et al., 2012). This highlights the importance of assessing the effect(s) of pesticides on *A. aquaticus* via direct and indirect effect pathways.

In this context, the objectives of the present study were (i) to assess fungicide-induced shifts in the leaf-associated microbial community and (ii) to unravel the biological relevance of food-quality and waterborne related toxicity of a fungicide on the food processing (consumption and excretion) as well as the physiological fitness (FA composition, lipid content; cf., Koop et al. 2011) and growth of *A. aquaticus*. Because triazole fungicides can negatively affect both fungi and animals (Stenersen, 2004), epoxiconazole was chosen as a model fungicide at a concentration inducing sublethal effects on the test organism *A. aquaticus* (i.e., 15 µg/L; EC₂₀ = 15.7 µg/L, see Supplementary data). The use of epoxiconazole at this particular concentration is further motivated by its extensive usage for crop protection (Hillocks, 2012) and its frequent detection in surface waters (e.g., Passeport et al., 2013) and edge-of-field run-offs at maximum concentrations up to 6 µg/L (Liess and von der Ohe, 2005) and 20 µg/L (Neumann et al., 2002), respectively. Using a 2x2-factorial design, epoxiconazole was applied either during the microbial conditioning of the leaf

material or during the bioassay with *Asellus* similar to the method in Zubrod et al. (2015a). Under this test design, *A. aquaticus* was affected either indirectly via the food, directly over the water phase, or combined via both pathways (i.e., food and water phase). To characterize the fungicide-induced effects on the conditioning status and thus the nutritional quality of the leaf material, fungal biomass, bacterial abundance, microbial C usage, and FA composition were assessed. Moreover, changes in food processing as well as the physiological fitness and growth of *A. aquaticus*, induced by the different effect pathways, were determined. We hypothesized that epoxiconazole modifies the leaf-associated microbial community, ultimately affecting the physiology of the model species (indirect effect pathway). Additionally, we hypothesized direct effects of epoxiconazole on *A. aquaticus* via the water phase based on the results of the preliminary experiment (direct effect pathway; Supplementary data). Based on the results of Zubrod et al. (2015a), it was further assumed that both pathways interact when applied in combination.

2. Materials and methods

2.1 Chemicals

The commercially available fungicide-formulation OPUS[®] (125 g epoxiconazole/L; BASF SE, Ludwigshafen am Rhein, Germany) was used to avoid any further addition of organic solvents. According to the 2x2-factorial test design, epoxiconazole was applied on two occasions: first, during the conditioning process of leaf material serving as food for *A. aquaticus* and, second, during the bioassay on the different effect pathways. To accomplish this, OPUS[®] was diluted either in an artificial nutrient medium (i.e., conditioning medium; Dang et al., 2005) or in filtered water from the sampling site of the model shredder (i.e., test medium) during the set-up or water exchanges of the conditioning runs and the bioassay, respectively, to achieve a nominal concentration of 15 µg epoxiconazole/L in the water phase. If not further specified, chemicals (analytical grade) were purchased from Roth, Sigma-Aldrich, or VWR.

2.2 Sources of the leaf material, microorganisms and asselids

Black alder leaves (*Alnus glutinosa* (L.) Gaertn.) were collected from trees in the surroundings of Landau, Germany (49°12'01" N; 08°05'40" E), in autumn 2012 shortly before abscission and stored at -20 °C until further processing. Leaf material was generally pre-conditioned in the field as per Zubrod et al. (2011) to obtain a near-natural microbial community for the conditioning of food used in the bioassay. Briefly, leaves were deployed in the Pinglaström, a near-natural stream close to Knivsta, Sweden (59°46'19" N; 17°45'19"E), for 14 d using nylon fine-mesh leaf bags (mesh size = 0.5 mm; 10 leaves per bag; step I in Fig. 1). After 14 d the leaf material was supplemented with unconditioned leaf material in the laboratory and kept in

aerated conditioning medium in darkness at 15 ± 1 °C for an additional seven days (step II in Fig. 1). By this approach, different stages of conditioning and thus an assumed high diversity of aquatic hyphomycetes was achieved (cf., Gessner et al., 1993).

One week prior to the start of the bioassay, *A. aquaticus* were collected by kick-sampling in a shallow pond in Uppsala, Sweden (59°48'49" N; 17°39'57" E), situated in an area where green belts and forestry were the most prominent types of land-use (Table S1). This limited the chance for a pre-exposure of the test organisms towards pesticides. Asselids of approximately 6 to 8 mm body length (measured from the base of the antennae to the back end of the operculum) were selected in the laboratory; organisms displaying visual evidences of acanthocephalan parasites were excluded to reduce variability during the bioassay (Dezfuli et al., 1994; Green et al., 1986). Test organisms were acclimated in aerated test medium to the experimental conditions, i.e. darkness at 15 ± 1 °C in a temperature-controlled room. During this acclimation phase, asselids were fed *ad libitum* with pre-conditioned black alder leaves. In order to assess the initial lipid content of asselids, randomly chosen individuals were shock-frozen in liquid nitrogen during the set-up of the bioassay and stored individually at -80°C until further processing. Simultaneously to the sampling of *Asellus*, pond water samples were taken for water quality analysis (Table S2).

2.3 Bioassay

The 2×2-factorial test design (see Zubrod et al., 2015b, for details) resulted in four different factor combinations (i.e., treatments), each consisting of 40 replicates at test initiation: (i) media free from epoxiconazole during leaf-conditioning and bioassay (control), (ii) application of epoxiconazole during leaf-conditioning but not

during the bioassay (indirect), (iii) application of epoxiconazole during the bioassay but not during leaf-conditioning (direct), and (iv) application of epoxiconazole during both leaf-conditioning and bioassay (combined).

To ensure a constant quality of the leaf material used as food during the 28-day-lasting *Asellus* bioassay, an independent run of leaf conditioning was set up for each of the four weeks. Terminations of the individual conditioning runs thereby coincided with the weekly medium exchange in the bioassay (see below). For each conditioning run, leaf strips of up to 45 cm² were cut from thawed black alder leaves and sewed into nylon fine-mesh leaf bags (6 x 10 cm; mesh size = 0.5 mm; step III in Fig. 1). Leaf strips were conditioned for 10 days (i.e., one conditioning run) in circular aquaria ($n = 3$; 75 strips per aquarium) containing 5 L aerated conditioning medium in the absence and presence of epoxiconazole at 15 µg/L. Thereby, 30 g pre-conditioned black alder leaves (fresh weight after blot-drying; ± 0.5 g) per aquarium served as microbial inoculum (step IV in Fig. 1). Conditioning was performed in a temperature-controlled room at 15 ± 1 °C in darkness. After conditioning, one leaf disc (diameter = 20 mm) was cut from each side of the midrib using a cork borer and immediately introduced into the bioassay (step V in Fig. 1). At the same time ten leaf strips randomly chosen from the conditioning aquaria were preserved for analyses of the leaf-associated microbial community as well as FA analysis (see chapter 2.4 for details).

Each replicate of the *Asellus* bioassay consisted of a 250-mL glass beaker containing 200 mL test medium with the respective epoxiconazole concentration (step VI in Fig. 1). Within each beaker, a single *A. aquaticus* and two leaf discs, originating from two different leaf strips, were situated in a cylindrical stainless steel cage (height = 8.0 cm; diameter = 5.5 cm; mesh size = 0.5 mm). This setup allowed for a careful

transfer of the test organisms into new beakers during the weekly medium exchange and avoided coprophagy. In addition to the leaf material accessible for feeding, the two corresponding leaf discs originating from the same leaf strips were situated beneath the cylindrical cage. These additional leaf discs were separated from the cylindrical cage by a watch glass (diameter = 6.0 cm), situated on pre-combusted pebbles, ensuring a 0.5 cm gap between the watch glass and the beaker and preventing an interaction between the leaf material and asselids' feces. Given that these leaf discs were not accessible for feeding by *Asellus*, quantification of microbial and abiotic leaf mass loss was possible individually for each beaker. Beakers were randomized in a temperature-controlled room at 15 ± 1 °C and aerated individually throughout the study, while the experiment was run in darkness.

Every seventh day, the test medium containing the respective concentration of epoxiconazole and the leaf discs were renewed. Therefore, remaining leaf material and any shredded leaf tissue were retrieved from the test beakers. Leaf discs accessible for and protected from feeding were dried separately to constant weight at 60 °C and afterwards weighed to the nearest 0.01 mg to assess the leaf mass loss driven by asselids' shredding activity (Zubrod et al., 2011). The excretion by asselids was measured individually for each replicate by filtering the old medium over pre-combusted and pre-weighed glass fiber filters (pore size 1-3 µm; GF/6, Whatman GmbH, Germany) to retain all feces quantitatively. Filters containing fecal pellets were dried and weighed as described for the leaf discs. Feces production was not quantified for the first week, since feces may have originated from food ingested prior to the bioassay (cf. Bärlocher and Kendrick, 1975). At the time of the water exchange, samples were taken for water quality analysis. Measurements did not reveal substantial differences in the assessed parameters among treatments (Table S2,

Supplementary data), indicating this source of variation was negligible for the outcome of the study. Dead animals were counted and discarded from the test system during the weekly water exchange and withdrawn from statistical analyses on asselids' food processing, physiological fitness, and growth. At the end of the bioassay (i.e., after 28 d), all remaining animals were carefully blot-dried, shock-frozen in liquid nitrogen and stored individually at -80 °C until further analyses.

2.4 Analyses of the leaf-associated microbes

The leaf-associated content of ergosterol (i.e., a membrane-bound molecule predominantly produced by true or higher fungi; sensu latu Frostegård and Bååth, 1996) was used as a proxy to estimate the fungal biomass (Gessner, 2005). In brief, after lyophilization of the leaf samples ($n = 16$; four conditioning runs á four samples; 50-100 mg per sample), ergosterol was extracted in alkaline methanol followed by purification using solid-phase extraction (Sep-Pak[®] Vac RC tC18 500 mg sorbent, Waters, Milford, MA, USA). By applying 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 ergosterol content was quantified at 282 nm and normalized to the initial sample weight.

Bacterial abundance associated with the leaf material was determined according to Büsing (2005). Briefly, one leaf disc (diameter = 10 mm) was cut from each of ten randomly chosen leaf strips, pooled into one replicate ($n = 16$; four conditioning runs á four samples) and preserved in 2% formalin buffered with 0.1% sodium pyrophosphate (v:w). Bacteria were detached from the leaf discs using ultrasonication. Subsequently, sample aliquots of 10 µL were filtered over aluminum

oxide filters (0.2 μm pore size; Anodisc, Whatman, Beckham, UK) and retained cells were stained using SYBRGreen II (Molecular Probes, Eugene, OR, USA). Digital photographs were taken by applying fluorescence microscopy to determine the mean number of bacterial cells on 20 microscopic fields per sample (Axio Scope.A1, AxioCam MRm and Axio-Vision Rel. 4.8, Carl Zeiss MicroImaging, Jena, Germany). Bacterial counts were dry weight-normalized using another set of ten leaf discs originating from the same leaf strips.

Effects on the microbial capacity to metabolize different C-substrates (i.e., functioning of aerobic C-using microbes) were assessed using Biolog EcoPlates™ (Biolog, Hayward, CA, USA). These plates contain 31 C-substrates, classified into six guilds (carbohydrates, carboxylic acids, amino acids, complex carbon sources, phosphate carbon, and amines), preloaded and dried in 96-well microwell plates (Table S4). The C-substrate metabolization by microorganisms is thereby indicated by a color reaction using tetrazolium violet as a redox dye. Samples were generally processed as described in Maul et al. (2006), with slight alterations. Briefly, one leaf disc (diameter = 10 mm) was cut from each of six randomly chosen leaf strips and pooled into one replicate ($n = 24$; four conditioning runs á six samples). Leaf discs were placed in sterilized Erlenmeyer flasks (E-flasks) containing 10 mL sterile, deionized water. The E-flasks were placed on an orbital shaker set at 75 rpm in complete darkness for 2 h. Afterwards, the E-flasks were vortexed twice for 30 s to detach leaf-associated microbes. Sample aliquots of 100 μL were transferred into each well of the EcoPlates™. Plates were covered with lids and incubated at 15 ± 1 °C for 96 h, while the absorbance at 540 nm was measured every 12 h using a microplate reader (Plate Chameleon, Hidex, Turku, Finland). In order to avoid false positive results, a threshold absorbance value of 0.25 after blank correction was set for the

analyses on the richness of used C-substrates, defined as the number of oxidized substrates (cf. Garland, 1996).

2.5 Fatty acid analysis

The extraction of asselids' lipids ($n = 10$ for each treatment of the bioassay as well as organisms sampled at test initiation) was conducted in a 3:2 hexane:isopropanol solution (v:v) according to Lau et al. (2012) after lyophilization of the organisms. For leaf samples ($n = 12$; four conditioning runs á three complete leaf strips), lipids were extracted in a 2:1 chloroform:methanol (v:v) solution as per Folch et al. (1957). After extraction, all samples were processed equally following the protocol of Lau et al. (2012). The total lipid content of each extract was determined by weighing to the nearest 0.1 μg . Therefore, 10 μL of the extract were transferred to a pre-weighed aluminum weighing pan, the solvent was evaporated on a heating plate and the remaining lipids were weighed on a microbalance. This procedure was conducted five times for each extract and the resulting lipid content was averaged. Afterwards, extracts were saponified using NaOH in dry methanol and methylated with 14% methanolic BF_3 solution to convert FAs into fatty acid methyl-esters (FAME). Concentrations of the individual FAME were measured using a GP3800 gas chromatograph (Varian AB, Stockholm, Sweden) equipped with a flame ionization detector. To identify and quantify FAME peaks, the GLC-461 FAME mixture (NuChek Prep, Elysian, Minnesota, USA) and the internal standard methyl 15-methylheptadecanoate (Larodan, Malmö, Sweden) were used, respectively. Detailed descriptions on the capillary column, injection conditions and temperature program can be found in Lau et al. (2012). The detection limit of FA masses was approximately 0.1 ng.

2.6 Measurement of fungicide concentrations

Triplicate 15-mL water samples were taken approximately 10 min after fungicide application and at the termination of the condition run (after 10 days) or prior to the water exchange in the bioassay (after 7 days) and stored at -20 °C until the measurement of fungicide concentrations. Measurements were generally performed according to Zubrod et al. (2015a), using an ultra-high performance liquid chromatography-mass spectrometry system equipped with a benchtop orbitrap system (all Thermo Fisher Scientific, Bremen, Germany) running in full scan mode (scan range 100-2000 m/z). Samples were directly injected, concentrated on-line and epoxiconazole was identified and quantified using the accurate mass of m/z 330.0803 $[M+H^+]$ and an external calibration ranging from 1 to 80 $\mu\text{g/L}$, respectively. Limit of detection and limit of quantification were calculated according to Miller & Miller (2002). Measured initial and nominal fungicide concentrations complied well with a maximum deviation of 8% (Table 1), indicating an appropriate dosage of the fungicide and justifying the use of nominal concentrations throughout this paper.

2.7 Statistical analyses

Data on food processing (i.e. consumption and excretion) and growth of *A. aquaticus* were quantified as detailed in Zubrod et al. (2015b). For univariate analyses, normal distribution and homoscedasticity of the data were checked using visual inspection and Levene's test, respectively. Student's t -tests were performed for variables assessing the conditioning status of the leaf material (i.e., ergosterol content, number of bacterial cells, and richness of C-substrate usage). Food processing as well as asellids' lipid content and growth were evaluated by two-factorial ANOVAs to

judge statistical significance of the assessed factors and their interaction. Differences in the mortality between treatments were addressed by pairwise comparisons using chi-square tests followed by Bonferroni-Holm corrections for multiple comparisons.

The conventional application of null hypothesis significance testing (NHST), however, is afflicted with two main points of criticism (Nakagawa and Cuthill, 2007). First, the *P*-values only give information on the probability of the test results or more extreme results given the null hypothesis is true, and not the probability of that null hypothesis given the data. Second, NHST lacks information about the effect size. Therefore, relative effect sizes as percentage change compared to the control treatment (Kaiser, 1989) are provided irrespective of statistical significance wherever mathematically calculable and biologically sensible. Furthermore, since the biological relevance of an effect (i.e., an effect considered as important for environmental health) and its respective statistical significance are not necessarily linked (EFSA, 2011), the discussion of this paper reports, but is not solely based on, the statistical significance of the results.

C-substrate metabolization of the EcoPlates™ was calculated by subtracting the absorbance of the blank from that of each corresponding C-substrate. The blank-corrected absorbance was ranked across all substrates within each replicate and plotted to visualize differences between the two treatments over time on a broader scale (i.e., without distinction between carbon guilds). Furthermore, time-series analyses for the usage of the six C-guilds were performed by fitting adequate models to the absorbance data from EcoPlates™. The models fitting the data best were selected based on visual judgment and Akaike's information criterion (see Table S3 for the selected models and their parameterization). Median absorbance and

associated standard errors were compared between treatments to test for significant differences in C-guilds' metabolization.

For the data on FA composition, multivariate homogeneity of group dispersions (i.e., variances) was checked. Significant shifts in the composition of FAs were analyzed with permutational multivariate analyses of variance (PERMANOVAs), performed on square-root-transformed data to reduce the effect of dominant groups (Clarke and Warwick, 2001) and applying Bray-Curtis dissimilarities as a distance measure between groups. In case of multiple comparisons, *P*-values were adjusted using the Benjamini-Hochberg method. Furthermore, non-metric multidimensional scaling (NMDS) ordination plots (Clarke, 1993) were prepared from resemblance matrices of Bray-Curtis dissimilarities among analyzed groups for a graphical interpretation of the observed dissimilarities among treatments.

Statistical analyses and figures were accomplished using the open source software R (version 3.0.2 for Mac OS X, 10.9; R Development Core Team, 2015) supplemented by the add-on packages “drc” (Ritz and Streibig, 2005), “plotrix” (Lemon, 2006), and “vegan” (Oksanen et al., 2015). The level for statistical significance was set at $P < 0.05$ and the term “significant(ly)” is exclusively used in the sense of “statistical significance” in the following.

3. Results & discussion

3.1 Effects on microbial communities and the nutritional quality of the leaf material

As hypothesized, epoxiconazole-induced alterations in leaf-associated microbial communities, namely fungal biomass and bacterial abundance, were found after 10 days of conditioning, i.e. before being introduced into the bioassay. Fungal biomass, measured as ergosterol, was significantly reduced by ~25% compared to the control ($P = 0.033$; Table 2). Fungicide-induced reductions in this sum parameter are usually associated with shifts in the community composition of aquatic hyphomycetes, such as reported for slightly higher concentrations of another triazole fungicide (i.e., tebuconazole; Zubrod et al., 2015c). Given that epoxiconazole is up to eight-fold more effective against aquatic fungi than tebuconazole (Dijksterhuis et al., 2011), shifts in the leaf-associated fungal community composition, although not measured, likely also occurred in the present study. Similarly to the fungal biomass, bacterial abundance was slightly and non-significantly reduced by approximately 15% ($P = 0.268$; Table 2) – a pattern also reported for tebuconazole-treated leaf material (Zubrod et al., 2015c). Since bacteria seem to be incapable of producing ergosterol (*sensu latu* Frostegård and Bååth, 1996), the reduced number of bacterial cells cannot be linked to the observed reduction of ergosterol.

The functional properties of the microbial communities assessed by means of EcoPlate™ analyses, however, supported the conjectured shift in the microbial community composition. Results showed an almost consistently higher metabolization for all C-substrates in the fungicide treatment than in the control at most of the assessed time points (Fig. S2, Supplementary data). In other words, the richness of used C-substrates was significantly higher for exposed communities than for those in the control ($P = 0.011$). In particular, carbohydrates, carboxylic acids, and

amino acids were the C-guilds contributing most to the observed differences, as after 72 hours the metabolization for complex carbon sources, phosphate carbon, and amines were again similar for both treatments (Fig. 2; Table S4, Supplementary data). These findings are supported by Thompson et al. (2015), who showed a higher overall metabolization of carbohydrates and amino acids at stream sites affected by a pesticide spill when compared to uncontaminated reference sites. This pattern of higher C-metabolization under pesticide exposure is most likely linked to a contaminant-induced community shift of microbes favoring generalists (Duarte et al., 2008; Ratcliff et al., 2006) ultimately altering the kinetics of carbohydrases (e.g., β -glucosidase, β -xylosidase, and cellobiohydrolase) given the interspecific variance (as reviewed in Lynd et al., 2002). These processes may in turn influence leaf litter breakdown rates over the long term (cf. Artigas et al., 2012).

Although it is generally accepted that the community composition of leaf-associated microorganisms determines the nutritional quality of leaf material for shredding invertebrates (as reviewed in Bärlocher and Sridhar, 2014), the factors driving this pattern remain unclear (Rong et al., 1995). One possible explanation is the capability of aquatic hyphomycetes to produce polyunsaturated FAs (PUFAs; Arce Funck et al., 2015) that are easily incorporated in consumer tissue, in contrast to bacteria, which are rich in branched FAs that are likely catabolized by consumers (Kaneda, 1991). Epoxiconazole-exposure did not significantly affect the overall composition of FAs ($n = 12$; $P = 0.279$; Fig. 3a) and the composition of all essential PUFAs (i.e., α -linolenic acid (ALA), 18:3 ω 3; linolenic acid (LA), 18:2 ω 6; eicosapentaenoic acid (EPA), 20:5 ω 3; docosahexaenoic acid (DHA), 22:6 ω 3; arachidonic acid (ARA), 20:4 ω 6; Parrish, 2009; $n = 12$; $P = 0.383$; Fig. 3b). Leaves exposed to epoxiconazole showed, however, a higher contribution of ARA (~55%; P

= 0.025) and EPA (~50%; $P = 0.037$) than those in the control (Table S5), despite the observed lower fungal biomass and bacterial abundance. Such shifts in the composition of certain PUFAs may be attributed to shifts in the aquatic hyphomycete community given the high variability in the FA composition among individual species (Arce Funck et al., 2015). Additionally, trophic upgrading, which is an improvement of the dietary availability of PUFAs and/or *de novo* synthesis of essential FAs by the microfauna associated with the leaf material, may have contributed to the observed shifts. Therefore, epoxiconazole-exposed leaf material seems to be of higher nutritional quality than the control leaves when solely based on FAs. This conjecture seems to be driven by the higher absolute concentration of ARA (~65%; $P = 0.003$) and EPA (~85%; $P = 0.015$), as those PUFAs can be seen as indicators for high food quality for invertebrates (Ahlgren et al., 2009).

3.2 Knock-on effects on *A. aquaticus*

Asellus exposed directly to epoxiconazole via the water phase reduced their consumption of leaf material significantly (~5%) and their excretion non-significantly (~10%; Fig. 4; Table 3) compared to the control, suggesting a slightly increased assimilation of the ingested food (~5%). When affected via the indirect pathway, asselids' showed non-significant trends to slightly increased leaf consumption (~5%) and reduced excretion (~20%; Fig. 4; Table 3), which indicates 25% increased assimilation relative to the control. This increased assimilation may point at an enhanced utilization of the ingested food, particularly when affected by indirect pathways (cf., Zubrod et al., 2015a), suggesting asselids build up higher energy reserves (=lipids) or grow faster. However, *Asellus* displayed non-significantly but biologically relevant lower growth rates when affected directly (~40%) and

indirectly by epoxiconazole (~30%; Fig. 5a; Table 3). Similarly, *Asellus*' overall lipid content was lower when affected via the direct (~20%) and indirect pathway (~10%; Fig. 5b; Table 3) compared to the control. In line with these observations, the realistic worst-case scenario, during which asselids experienced both effect pathways simultaneously, resulted in an interaction of the two effect pathways: non-significant reductions in leaf consumption (~15%) and excretion (~15%; Fig. 4; Table 3) were observed, while assimilation was unaffected. Nonetheless, growth (Fig. 5a; Table 3) and lipid content (Fig. 5b; Table 3) of asselids were 45% and 30%, respectively, lower than in the control. These data suggest an increased energy demand under toxic stress associated with detoxification processes (Maltby, 1999) when exposed directly over the water phase or due to the potential co-ingestion of epoxiconazole adsorbed to the leaf material (see calculations in the Supplementary data). The enhanced assimilation of the ingested food, however, did not fully compensate for the additional energy required, as growth and lipid reserves were partly substantially reduced (up to 45%; cf. Zubrod et al., 2011). Finally, leaf material conditioned in the presence of epoxiconazole may have had a lower nutritional quality triggered by the lowered biomass and the community shift of leaf-associated microbes, which are two important factors in the nutrition of *Asellus* (Graça et al., 1993; Graça et al., 1994). The higher contributions of essential PUFAs to the FA composition, on the other hand, seemingly did not compensate for the lower availability of microbial biomass and therefore the lower quality of the offered food. The energetic imbalance in *Asellus* affected via an altered quality of the food (indirect and combined pathway) finally seemed to affect the mortality compared to the control. While asselids' mortality remained on the control level when exposed via the waterborne pathway ($P = 1.000$), non-significant increases of 10% ($P = 0.618$) and 20% ($P = 0.098$) were

observed for the indirect and combined pathways, respectively (cf., Flores et al., 2014).

Although the overall lipid content of *Asellus* is considered a useful indicator to detect energetically costly effects on its physiological fitness (Koop et al., 2011), information on the composition of FAs may allow for a more mechanistic interpretation: the analysis showed significant shifts in FA compositions in affected *Asellus* relative to the control for the indirect ($P = 0.039$) and direct pathway ($P = 0.014$) as well as the combined scenario ($P = 0.003$; Fig. 6). Individual FAs revealed reduced concentrations in affected *Asellus* compared to control organisms irrespective of the effect pathway (Table S6). On the one hand, individual saturated FAs showed an up to ~60% reduction or almost depletion (i.e., 13:0 and 22:0; Tables S5 and S6) indicating their usage for catabolic processes to provide additional energy by β -oxidation (as reviewed in Tocher, 2003). On the other hand, peroxidation due to oxidative stress could explain the observed negative trend for some monounsaturated FAs and PUFAs in organisms subjected to the different effect pathways (Table S6; as reviewed in Halliwell and Gutteridge, 1984). Such declines in FAs can become crucial when reducing the concentrations of essential PUFAs (and their precursors) fundamental for life history traits of invertebrates (Parrish, 2009). In this regard, results revealed a non-significant tendency towards lower concentrations of ARA (~10-15%) and EPA (~10%) for asselids affected by epoxiconazole, again irrespective of the effect pathway (Table S6). Precursors for ARA and EPA were, conversely, found with up to 40% higher concentrations in affected *Asellus* compared to control organisms (Table S6), likely due to differences in elongation and desaturation rates (cf., Ahlgren et al., 2009).

4. Conclusions

The present study shows that epoxiconazole can provoke complex effects on decomposer-detritivore-systems and hence on the energy provisioning by heterotrophic processes in freshwater food webs. Since the present study used the relatively tolerant *A. aquaticus* as test species, the results obtained here may underestimate effects for more sensitive invertebrate species, despite similar effect patterns (e.g., Zubrod et al., 2015a). Although the direct water-exposure pathway generally provoked stronger effects than the diet-related pathway (Fig. 4, 5, and 6), both pathways are of biological relevance from a food web perspective. Reduced excretion of fecal pellets, for instance, may limit the availability of fine-particulate organic matter (FPOM), a high quality food source for collectors and filter feeders (Bundschuh and McKie, in press). Additionally, the reduction in the lipid content of asselids as well as the reduced contents of ARA and EPA may affect their growth, fitness, and reproduction (Ahlgren et al., 2009; Sparkes et al., 1996), with implications in local population sizes and consequences for organic matter processing. Finally, *A. aquaticus* represents one pathway for FAs, nutrients, and C from primary producers and leaf detritus towards higher trophic levels (e.g.; Lawton, 1970; Estlander et al., 2010). Therefore, implications on asselids' growth and reproduction influence the energy availability for higher trophic levels. More importantly, a trophic transfer of essential ≥ 20 -C PUFAs is crucial for the growth and development of organisms at higher trophic levels (Kainz and Fisk, 2009; Lau et al., 2012). Contaminant-induced shifts in the FA composition of primary consumers, as observed during the present study (Table S6), therefore may have far-reaching consequences in aquatic food webs.

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Conflict of interest

The authors declare that they have no conflict of interest regarding the material discussed in the manuscript.

Appendices

Supplementary data is available in the online version of this article and contains the following: (i) description and results of the preliminary experiment, (ii) GIS-analyses of the land-use pattern around the sampling site of *A. aquaticus*, (iii) water quality parameters of the test medium, (iv) results of rank analyses of C-substrate metabolization, (v) description of the models and respective parameterization for time-series analysis of C-guilds' metabolization, (vi) absorbance data of EcoPlates™ and statistical analyses for each time point, (vi) fatty acid composition of conditioned leaf material, (vii) calculation of epoxiconazole possibly adsorbed to the leaf material, and (viii) fatty acid composition of *A. aquaticus*.

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Fig. 1: Schematic illustration detailing the experimental design of the present study (see chapters 2.2 and 2.3; adapted after Zubrod et al., 2015b; Zubrod et al. 2015c): (I) pre-conditioning of fresh leaf material in the field, (II) pre-conditioned and fresh leaf material kept in conditioning medium to generate microbial inoculum for the bioassay, (III) leaf strips cut from fresh leaf material and sewed into nylon fine-mesh bags, (IV) conditioning of leaf strips together with 30 g microbial inoculum in absence and presence of epoxiconazole (15 µg/L), (V) cutting of leaf discs used as food for the bioassay and analyses of leaf material-associated microbes, and (VI) bioassay to unravel the relevance of food-quality related and waterborne toxicity of epoxiconazole (15 µg/L).

Fig. 2: Time-series analyses of absorbance measurements for the six carbon guilds metabolized by microbes over the incubation period of 96 h ($n = 24$). Bars represent standard errors around medians (filled circles). Control and epoxiconazole treatment are displayed in black and grey, respectively, with asterisks indicating significant differences between them.

Fig. 3: Non-metric multidimensional scaling (NMDS) ordination (with 95% confidence ellipses [CEs]) for the composition of (a) all detected fatty acids, and (b) essential fatty acids (i.e. ALA, LA, EPA, DHA and ARA) associated with the leaf material ($n = 12$) conditioned under control conditions (black filled circles) and exposed to 15 µg epoxiconazole/L (grey filled circles). The stress values are displayed as a “goodness-of-fit” measure of the NDMS, with values below 0.2 indicating a reasonable fit (Clarke, 1993).

Fig. 4: Mean ($\pm$ 95% CI) leaf consumption (●) and excretion (▲) of *A. aquaticus*, expressed in percent relative to the consumption and excretion in the epoxiconazole-free control, respectively, subjected for 28 days to four treatments: the control (epoxiconazole-free), the indirect (diet-related effects), the direct (waterborne toxicity) and the combined exposure scenario ($n = 37/33/38/29$). The statistical significances of single variables and their interactions are detailed in Table 3.

Fig. 5: Mean ($\pm$ 95% CI) (a) growth ($n = 37/33/38/29$) and (b) lipid content ($n = 10$) of *A. aquaticus* being subjected to the control, the indirect (diet-related effects), the direct (waterborne toxicity) and the combined exposure scenario over 28 d. The solid, horizontal line in Fig. 5b indicates the mean initial lipid content of *A. aquaticus* ($\pm$ 95% CI; indicated by dashed, horizontal lines) at the set-up of the bioassay ($n = 10$). Statistical significances of single variables and their interactions are detailed in Table 3.

Fig. 6: Non-metric multidimensional scaling (NMDS) ordination (with 95% CEs) of *A. aquaticus*' fatty acids composition ($n = 10$) including the control (black filled circles; solid, black line), the indirect (light grey squares; dashed, light grey line), the direct (light grey triangles; solid, light grey line) and the combined exposure scenario (dark grey diamonds; solid, dark grey line). The stress value is displayed as a “goodness-of-fit” measure of the NDMS, with a value below 0.2 indicating a reasonable fit (Clarke, 1993).

Table 1

Table 1: Nominal and mean measured concentrations of epoxiconazole ($\pm$ 95% confidence intervals [CI]; $n = 3$) in the conditioning and the bioassay medium. For both media, measurements were performed at the set-up (0 d) as well as shortly before the termination of the conditioning (10 d) and the weekly medium exchange (7 d).

Medium	Treatment	Time (d)	Nominal concentration ($\mu\text{g/L}$)	Measured concentration ($\mu\text{g/L}$)
Conditioning	Control	0	0.0	< LOD ^a
Conditioning	Control	10	0.0	< LOD
Conditioning	Direct	0	15.0	15.57 ± 0.63
Conditioning	Direct	10	15.0	2.2 ± 0.25
Bioassay	Control	0	0.0	< LOD
Bioassay	Control	7	0.0	< LOD
Bioassay	Indirect	0	0.0	< LOD
Bioassay	Indirect	7	0.0	< LOQ ^b
Bioassay	Direct	0	15.0	14.6 ± 5.98
Bioassay	Direct	7	15.0	12.23 ± 2.35
Bioassay	Combined	0	15.0	13.77 ± 3.31
Bioassay	Combined	7	15.0	15.97 ± 8.33

^aLimit of detection (LOD) = 0.23 $\mu\text{g/L}$ (~1.5% of the nominal concentration)

^bLimit of quantification (LOQ) = 0.46 $\mu\text{g/L}$ (~3.1% of the nominal concentration)

Table 2

Table 2: Mean ($\pm$ 95% CI) fungal biomass ($n = 15/13$) and bacterial abundance ($n = 16$) associated with the dry weight (dw) of the leaf material conditioned in the absence (=control) and presence (=direct) of 15 μg epoxiconazole/L. An asterisk denotes a statistically significant difference between the treatments.

Treatment	Fungal biomass (mg/g leaf dw)	Number of bacterial cells (10^8 cells/mg leaf dw)
Control	67.0 ± 13.2	2.6 ± 0.6
Direct	$51.0 \pm 7.9^*$	2.2 ± 0.5

Table 3

Table 3: Output for two-factorial ANOVAs, run for all *Asellus*-related variables. *P*-values printed in bold indicate statistical significance.

Variable	Source of variation	df ^a	Sum Sq ^b	Mean Sq ^c	<i>F</i> -value	<i>P</i> -value
Leaf consumption	Indirect	1	0.036	0.0360	0.289	0.5914
	Direct	1	0.515	0.5145	4.142	0.0438
	Indirect ×Direct	1	0.236	0.2359	1.899	0.1705
	Residuals	133	16.522	0.1242		
Excretion	Indirect	1	0.203	0.2033	2.478	0.118
	Direct	1	0.070	0.0701	0.854	0.357
	Indirect ×Direct	1	0.110	0.1105	1.347	0.248
	Residuals	133	10.583	0.0820		
Growth	Indirect	1	2.13	2.132	1.268	0.262
	Direct	1	4.20	4.203	2.500	0.116
	Indirect ×Direct	1	0.88	0.876	0.521	0.472
	Residuals	133	203.43	1.681		
Lipid content	Indirect	1	735.0	735.0	5.127	0.4471
	Direct	1	85.0	84.9	0.592	0.0303
	Indirect ×Direct	1	0.0	0.4	0.003	0.9576
	Residuals	40	4731	143.4		

^adf, degrees of freedom; ^bSum Sq, sum of squares; ^cMean Sq, mean squares

Figure 1

Figure 2

Figure 3a

Figure 3b

Figure 4

Figure 5a

Figure 5b

Figure 6