Diet-related effects of antimicrobials in aquatic decomposer-shredder and periphyton-grazer systems

Nahrungsbezogene Effekte von antimikrobiellen Substanzen in aquatischen Zersetzer-Zerkleinerer- und Periphyton-Weidegänger-Systemen

by

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Declaration

I hereby declare that I independently conducted the work presented in this thesis entitled "Diet-related effects of antimicrobials in aquatic decomposer-shredder and periphyton-grazer systems". 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, June 02, 2022

Place, Date Marco Konschak

This is a cumulative dissertation containing four published articles in peer-reviewed international journals.

Appendix A1: Konschak, M., Zubrod, J.P., Baudy, P., Fink, P., Kenngott, K.G.J., Lüderwald, S., Englert, K., Jusi, C., Schulz, R., Bundschuh, M., 2020. The importance of diet-related effects of the antibiotic ciprofloxacin on the leaf-shredding invertebrate *Gammarus fossarum* (Crustacea; Amphipoda). Aquat. Toxicol. 222, 1–10.

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Marco Konschak: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing - original draft. Jochen P. Zubrod: Conceptualization, Supervision, Writing - review & editing. Patrick Baudy: Conceptualization, Investigation, Writing - review & editing. Patrick Fink: Methodology, Resources, Writing - review & editing. Kilian G. J. Kenngott: Conceptualization, Methodology, Resources. Simon Lüderwald: Investigation, Writing - review & editing. Katja Englert: Investigation. Cynthia Jusi: Investigation. Ralf Schulz: Conceptualization, Funding acquisition, Resources, Writing - review & editing. Mirco Bundschuh: Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing.

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Appendix A4: Konschak, M., Zubrod, J.P., Duque Acosta, T.S., Bouchez, A., Kroll, A., Feckler, A., Röder, N., Baudy, P., Schulz, R., Bundschuh, M., 2021. Herbicide-induced shifts in the periphyton community composition indirectly affect feeding activity and physiology of the gastropod grazer *Physella acuta*. Environ. Sci. Technol. 55, 14699–14709.

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Abstract

Leaf-associated microbial decomposers as well as periphyton serve as important food sources for detritivorous and herbivorous macroinvertebrates (shredders and grazers) in streams. Shredders and grazers, in turn, provide not only collectors with food but also serve as prey for predators. Therefore, decomposer-shredder and periphyton-grazer systems (here summarized as freshwater biofilm-consumer systems) are highly important for the energy and nutrient supply in heterotrophic and autotrophic stream food webs. However, both systems can be affected by chemical stressors, amongst which antimicrobials (e.g., antibiotics, fungicides and algaecides) are of particular concern. Antimicrobials can impair shredders and grazers not only via waterborne exposure (waterborne effect pathway) but also through dietary exposure and microorganism-mediated alterations in the food quality of their diet (dietary effect pathway). Even though the relevance of the latter pathway received more attention in recent years, little is known about the mechanisms that are responsible for the observed effects in shredders and grazers. Therefore, the first objective of this thesis was to broaden the knowledge of indirect antimicrobial effects in a model shredder and grazer via the dietary pathway. Moreover, although freshwater biofilm-consumer systems are most likely exposed to antimicrobial mixtures comprised of different stressor groups, virtually nothing is known of these mixture effects in both systems. Therefore, the second objective was to assess and predict diet-related antimicrobial mixture effects in a model freshwater biofilm-consumer system. During this thesis, positive diet-related effects of a model antibiotic on the energy processing and physiology of the shredder *Gammarus fossarum* were observed. They were probably triggered by shifts in the leaf-associated microbial community in favor of aquatic fungi that increased the food quality of leaves for the shredder. Contrary to that, a model fungicide induced negative effects on the energy processing of *G. fossarum* via the dietary pathway, which can be explained by negative impacts on the microbial decomposition efficiency leading to a reduced food quality of leaf litter for gammarids. For diet-related antimicrobial effects in periphyton-grazer systems, a model algaecide altered the periphyton community composition by increasing nutritious and palatable algae. This resulted in an enhanced consumption and physiological fitness of the grazer *Physella acuta*. Finally, it was shown that complex horizontal interactions among leaf-associated microorganisms are involved, making diet-related antimicrobial mixture effects in the shredder *G. fossarum* difficult to predict. Thus, this thesis provides new insights into indirect diet-related effects of antimicrobials on shredders and grazers as well as demonstrates uncertainties of antimicrobial mixture effect predictions for freshwater biofilm-consumer systems. Moreover, the findings in this thesis are not only informative for regulatory authorities, as indirect effects and effects of mixtures across chemical classes are not considered in the environmental risk assessment of chemical substances, but also stimulate future research to close knowledge gaps identified during this work.

1 Introduction

1.1 Background

Microbial biofilms (assemblages of microbial cells associated with organic or inorganic surfaces; Romaní et al., 2013) play a fundamental role in the nutrient cycling of freshwater ecosystems (Sigee, 2006). For instance, detritus-associated microbial decomposers consisting mainly of bacteria and aquatic fungi substantially contribute to the mineralization of leaf litter (Hieber and Gessner, 2002) which represents an important allochthonous energy source for lotic systems (Fisher and Likens, 1973), and thus provide bound nutrients for stream communities (Marks, 2019). During microbial decomposition, leaf litter is chemically and structurally modified by the release of extracellular enzymes of bacteria and, in particular, aquatic fungi. This elevates the nutritional quality and palatability of leaf litter for detritivorous macroinvertebrates (i.e., shredders; Gessner et al., 1999), and thus exert a positive impact on their physiological fitness (Graça et al., 1993). Shredders, which are one of the four functional feeding groups of macroinvertebrates (collectors, grazers, predators and shredders; Vannote et al., 1980), are an important food source for higher trophic levels (MacNeil et al., 1999). Moreover, they transform leaf litter into fine particulate organic matter (e.g., feces), which serves as food source for collector organisms (Bundschuh and McKie, 2016).

Freshwater periphyton constitutes another important assemblage of microorganisms in rivers that mainly consists of algae (in particular diatoms and green algae) and bacteria building a submerged substrate-associated biofilm (Wetzel, 2001). Periphyton are key primary producers in stream ecosystems (Wu, 2017) and serve as a high-quality food source by providing essential nutrients, such as highly unsaturated fatty acids (HUFAs), to herbivorous macroinvertebrates (i.e., grazers; Guo et al., 2018). HUFAs play an important role for the development, somatic growth and reproduction of consumers, such as grazers (Brett and Müller-Navarra, 1997). Grazers are critical in aquatic ecosystems, since they control the biomass of periphyton (Rosemond et al., 2000) and are prey for predatory fishes (Dahl, 1998).

While detritus-associated microbial decomposers are essential for the nutrient supply in detritus-based stream food webs (Marks, 2019), periphyton constitutes a crucial food source in stream food webs in which detritus plays a minor role (Bunn et al., 2003; Delong and Thorp, 2006). Alterations in these biofilms by external factors may change their function

and thus indirectly affect the first consumer level (i.e., shredders and grazers) that strongly depends on these biofilms (e.g., Crenier et al., 2019). This, in turn, may have far-reaching consequences for other trophic levels (see above). Therefore, the integrity of these decomposer-shredder and periphyton-grazer systems is fundamental for intact heterotrophic- and autotrophic-based stream food webs.

However, both systems can be affected by chemical stressors, such as pharmaceuticals and pesticides (Feckler et al., 2015), which enter surface waters via point (e.g., wastewater treatment plant discharges) and non-point sources (e.g., (agricultural) surface runoff; Neumann et al., 2002; Kümmerer, 2009). In particular, antimicrobial substances (= antimicrobials), such as antibiotics, fungicides and algaecides used to control microbial pests and pathogens closely related to non-target bacteria, fungi and algae, respectively, are of particular concern (e.g., Brandt et al., 2015; Zubrod et al., 2019). By affecting non-target species in microbial communities via waterborne exposure, antimicrobials induce changes in the community composition (e.g., Pesce et al., 2006; Dimitrov et al., 2014). This, in turn, may not only alter microbial leaf-decomposition efficiency and primary production (Schmitt-Jansen and Altenburger, 2005a; Zubrod et al., 2015a), but could also change the food quality for shredders and grazers, potentially affecting their energy processing (= dietary intake and defecation) and physiology (i.e., food quality-related bottom-up (= FQ-B) effects of antimicrobials). For example, such responses were observed for crustacean shredders fed for 24 days with leaves that were exposed to fungicides during microbial conditioning (Zubrod et al., 2015c; Feckler et al., 2016). Besides potential dietary exposure effects due to fungicides that adsorbed to the leaves, the authors of these studies suggested that fungicides induced alterations in the leaf-associated fungal community in favor of less nutritional aquatic fungi, thereby reducing the quality of leaves and ultimately affecting the food assimilation and somatic growth of shredders. Contrary to fungicides, it is assumed that antibiotic-induced changes in the leaf-associated microbial community increase the quality of leaves for shredders, and thus lead to a higher somatic growth of this functional feeding group (Bundschuh et al., 2017). Similar to fungicides, algaecides (including herbicides that have algaecidal properties) seem to reduce the food quality of periphyton for grazers by replacing algae with less nutritional cyanobacteria ultimately affecting the physiological fitness of grazers (Rybicki and Jungmann, 2018). Therefore, in addition to direct effects of antimicrobials on shredders and grazers through waterborne exposure, they can also be affected via dietary exposure and FQ-B effects (dietary effect pathway, sensu Zubrod et al., 2015c).

1.2 Motivation and objectives

Despite strong evidence that antimicrobials can disrupt decomposer-shredder and periphyton-grazer systems (in this thesis summarized as freshwater biofilm-consumer systems) even at environmentally relevant concentrations (e.g., Zubrod et al., 2015c; Rybicki and Jungmann, 2018), little is known about the dietary effect pathway and, in particular, the mechanisms that induce FQ-B effects in both functional feeding groups. This is due to the fact that many studies suggesting FQ-B effects of antimicrobials on shredders and grazers used test designs that did not exclude direct application of the antimicrobial into the water phase (= waterborne exposure), which, however, can distort the interpretation of the results. This is especially the case for antibiotics and algaecides (e.g., Bundschuh et al., 2017; Rybicki and Jungmann, 2018), while at least for fungicides the employed test designs allowed a testing of both effect pathways (e.g., Feckler et al., 2016). Furthermore, none of these studies used biomarkers that traced potential FQ-B effects on shredders and grazers to demonstrate a direct link between microorganism-mediated changes in food quality and alterations in the physiology of the invertebrate. Therefore, the primary objective of the present thesis was to investigate if antimicrobials can induce FQ-B effects in shredders and grazers by using a proxy for changes in food quality, which can be tracked in the consumer. To achieve this, shredders and grazers were fed with microbial conditioned leaves and periphyton, respectively, colonized in the presences of antimicrobials, by omitting direct waterborne antimicrobial exposure. Additionally, concentrations were selected that induce clear effects on freshwater biofilms without having an impact on the respective consumer via waterborne exposure. This reduces the likelihood of direct antimicrobial effects in shredders and grazers, e.g. due to desorption of antimicrobials into the water phase. To demonstrate microorganism-mediated alterations in food quality for consumers, microbial community structures and the composition of FAs of the food for shredders and grazers were investigated. Furthermore, neutral lipid fatty acids (NLFAs) in consumers were assessed linking food quality changes with alterations in the consumers' physiological fitness. NLFAs are a promising tool for this purpose because they constitute not only important energy reserves in invertebrates (Azeez et al., 2014) but also reflect the diet of animals, and thus show changes in the abundance of essential FAs (Iverson, 2012), which are important for the animals' physiology (Brett and Müller-Navarra, 1997).

Moreover, previous studies exclusively focused on diet-related effects by antimicrobial mixtures containing mixture components of the same stressor group (i.e., only antibiotics, fungicides or algaecides). In reality, it is more likely that freshwater biofilm-

consumer systems are exposed simultaneously to these groups, as in surface waters pollutants occur in complex mixtures (Loos et al., 2013; Neale et al., 2020). Since the stressor groups have dissimilar effects on microbial communities when applied individually (see above), these antimicrobial mixtures could induce unpredictable effects in shredders and grazers via the dietary pathway. However, there is currently no knowledge about FQ-B effects on shredders and grazers by antimicrobial mixtures consisting of different stressor groups. Consequently, due to this data paucity, it cannot be excluded that these freshwater biofilm-consumer systems are inadequately protected by the current environmental risk assessment (ERA) in Europe. Therefore, the second objective of this thesis was to investigate effects of a model antimicrobial mixture on a freshwater biofilm-consumer system, and comparing the observed effects to effect predictions that are based on data from the individual mixture components collected during this thesis and previous studies.

The primary goal of this thesis is to contribute to closing the above-mentioned knowledge gaps by performing an in-depth assessment of diet-related antimicrobial effects in a decomposer-shredder system subjected to a model antibiotic, fungicide and a mixture of both, as well as a periphyton-grazer system exposed to a model algaecide. Therefore, the following research questions were addressed:

1. Do antimicrobials (i.e., antibiotics and fungicides) induce FQ-B effects in shredders by impacting their behavior, energy processing and physiology, as assumed in previous studies?
2. Can diet-related antimicrobial mixture effects in freshwater biofilm-consumer systems be predicted?
3. Can antimicrobials (i.e., algaecides) affect the nutritional quality of periphyton for aquatic grazers, and can these changes be reflected by an altered consumption and physiology?

2 Thesis layout and model system

2.1 Thesis layout

The present thesis is divided in two experimental phases (Fig. 2.1) addressing the three research questions in section 1.2 Motivation and objectives.

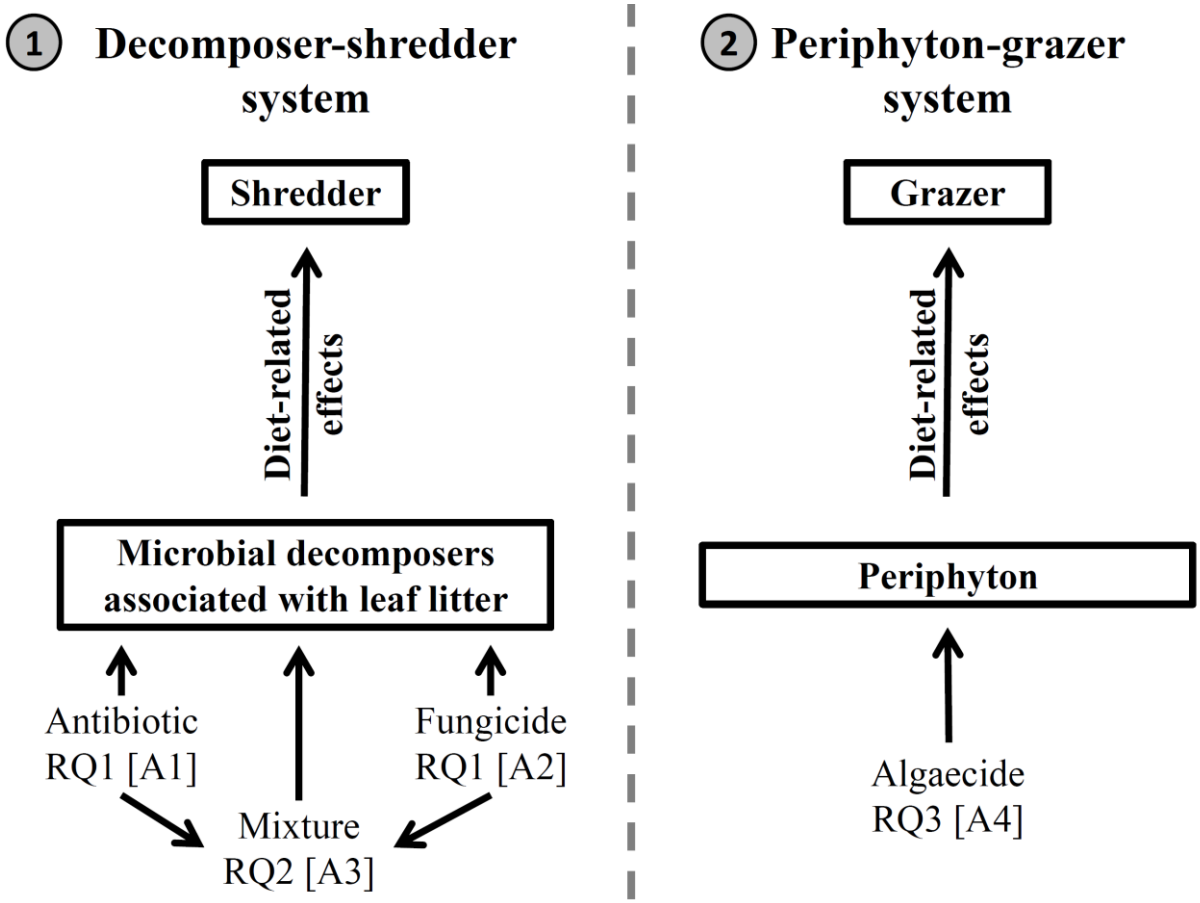

Fig. 2.1 Scheme illustrating the two phases (encircled numbers) of this thesis that investigate diet-related antimicrobial effects in decomposer-shredder and periphyton-grazer systems. Each research question (RQ1 – RQ3) is assigned to the respective chemical stressor group(s) and publication (**Appendix A1 – A4**).

2.2 Model antimicrobials and organisms

To answer the research questions, the following model antimicrobials were selected: the DNA gyrase and topoisomerase IV inhibitor ciprofloxacin (CIP), the quinone outside inhibitor azoxystrobin (AZO) and the photosystem II inhibitor diuron (DIU) were used as model antibiotic, fungicide, and algaecide, respectively, since they are frequently detected in European surface waters (partly up to the µg/L range) and pose a hazard to aquatic organisms

(e.g., Schreiner et al., 2016; Danner et al., 2019; Zubrod et al., 2019). Although the application of DIU (also used as herbicide; European Food Safety Authority, 2005) is nowadays restricted in Europe (Mohaupt et al., 2020), the algaecide was selected as enough literature is available to facilitate the understanding of potential FQ-B effects on grazers. Nominal concentrations of each substance selected in this thesis were verified by analyzing water samples via an ultra-high-performance liquid chromatography-mass spectrometry (cf. Zubrod et al., 2015c).

Moreover, a model decomposer-shredder and periphyton-grazer system was used. The former consisted of a near-natural leaf-associated microbial community sampled from the Rodenbach near Kerzenheim, Germany (49°33'N; 8°02'E) and the amphipod shredder *Gammarus fossarum* collected from the Hainbach near Frankweiler, Germany (49°14'N; 8°03'E). *Gammarus* spp. are key species in the leaf litter breakdown in low-order streams of the Northern Hemisphere (Piscart et al., 2009), and they are considered as useful test species for ecotoxicological studies as they are highly sensitive to chemicals and easy to handle in the laboratory (Kunz et al., 2010). The model periphyton-grazer system comprised a near-natural periphyton community scraped from the sun-exposed surface of stones that stem from the Eußerbach near Eußerthal, Germany (49°15´N; 7°57´E) and the gastropod grazer *Physella acuta* obtained from an established in-house culture in the iES Landau (University Koblenz-Landau). *P. acuta* is considered as suitable test organism for ecotoxicological studies as the gastropod grazer is highly abundant in freshwater ecosystems worldwide and also easy to handle in the laboratory (Horak and Assef, 2017). Note that complex near-natural microbial communities were used in this thesis instead of artificially assembled communities, as they cover all important taxonomic groups and consist of a variety of different species, which could only be achieved with great afford for the latter.

3 Experimental Phases

3.1 Phase 1: diet-related antimicrobial effects in decomposer-shredder systems

3.1.1 Diet-related antibiotic effects in shredders

The first part of phase one aimed at assessing indirect antibiotic effects on shredders via the dietary effect pathway. Using a three-tiered testing approach, firstly, the waterborne toxicity of CIP to *G. fossarum* was screened via a feeding activity assay (a well-established test design; e.g., Zubrod et al., 2014; Fig. 3.1a), in order to avoid CIP concentrations causing waterborne effects in animals during the main experiment. Therefore, the mortality and the consumption of leaf discs from black alder (*Alnus glutinosa*) by gammarids were quantified during a 7-day exposure towards different CIP concentrations (0, 0.5, 6.5, 12.5, 18.5, 24.5 mg/L).

In the second part of this phase, leaf material from black alder was microbially conditioned for 12 days in the absence and presence of different CIP concentrations (0, 20, 100, 500, 2500 µg/L) to assess microorganism-mediated alterations of the food quality for shredders by using a food choice assay (cf. Bundschuh et al., 2009; Fig. 3.1b). The food selection of *G. fossarum* when offered leaf material that was microbially colonized under control conditions or in the presence of CIP served as proxy for the leaf palatability for gammarids and, thus, indicates alterations in leaf quality (Bundschuh et al., 2011, Zubrod et al., 2015a). To achieve a more in-depth understanding of the mechanisms underlying observed effects on the food choice behavior of the amphipod shredder, microbial activity (i.e., microbial leaf decomposition) and the microbial community structure was determined. For the latter, the following microbial parameters were used: according to Gessner (2005), ergosterol, a proxy for fungal biomass, was analyzed by extracting and quantifying it via solid-phase extraction and high-performance liquid chromatography, respectively. Bacterial densities were measured using epifluorescence microscopy following Buesing (2005). Furthermore, the community composition of aquatic hyphomycetes (AH), an important fungal group that substantially increases the nutritional value of leaves for shredders during microbial conditioning (Bärlocher, 1985), was determined via their spore morphology (Pascoal and Cássio, 2004).

Lastly, in the third experiment (i.e., a 24-day long-term assay), diet-related effects on the energy processing and physiology of *G. fossarum* were investigated using a full-factorial study design established by Zubrod et al. (2015b) (Fig. 3.1c). The first factor was leaf material used as food source for *G. fossarum*, which was microbially conditioned for 12 days in the absence or presence of CIP (0.5 mg/L), and the second factor was the absence or presence of CIP in the water phase (0.5 mg/L). The latter served as reference to distinguish dietary-related effects from potential waterborne toxicity through CIP that desorbed into the water phase. Note that the combination of both factors was also investigated during the assay but will not be considered further here, since combined effects of the waterborne and dietary pathway are not relevant answering the three central questions of the present thesis. To assess long-term effects, the energy processing (i.e., leaf consumption and feces production) and somatic growth (cf. Zubrod et al., 2011) as well as energy reserves (NLFAs) of the gammarids were quantified. Moreover, NLFAs of the conditioned leaf material from black alder and the above-mentioned microbial parameters were determined to investigate FQ-B effects on gammarids. For the measurement of NLFAs, a method using gas chromatography with flame ionization (GC FID) was established during this phase at the iES Landau. Lipids of gammarids and leaf material were purified, according to Bligh and Dyer (1959). Afterwards, NLFAs were extracted via SPE and rapidly transesterified to fatty acid methyl esters (FAMEs) by using trimethylsulfonium hydroxide (cf. Butte, 1983). FAMEs were determined via GC FID following Fink (2013). NLFA concentrations were quantified by using an external standard calibration and corrected via extraction blanks and the recovery rate of an internal standard (deuterated 18:0 FA). For a detailed explanation of all experimental setups and analyses, see **Appendix A1**.

It was hypothesized that *G. fossarum* would be tolerant to waterborne CIP exposure due to its specific mode of action, namely to inhibit bacterial DNA gyrase (Hooper and Wolfson, 1988). CIP was, however, expected to negatively affect leaf-associated bacteria, and, consequently, to reduce the competitive pressure on aquatic fungi by bacteria (Gulis and Suberkropp, 2003; Schneider et al., 2010). This would lead to an enhanced fungal growth, and thus a higher palatability and food quality for the amphipod shredder (Bundschuh et al., 2009) resulting in an increased energy processing, somatic growth and energy reserves of *G. fossarum*.

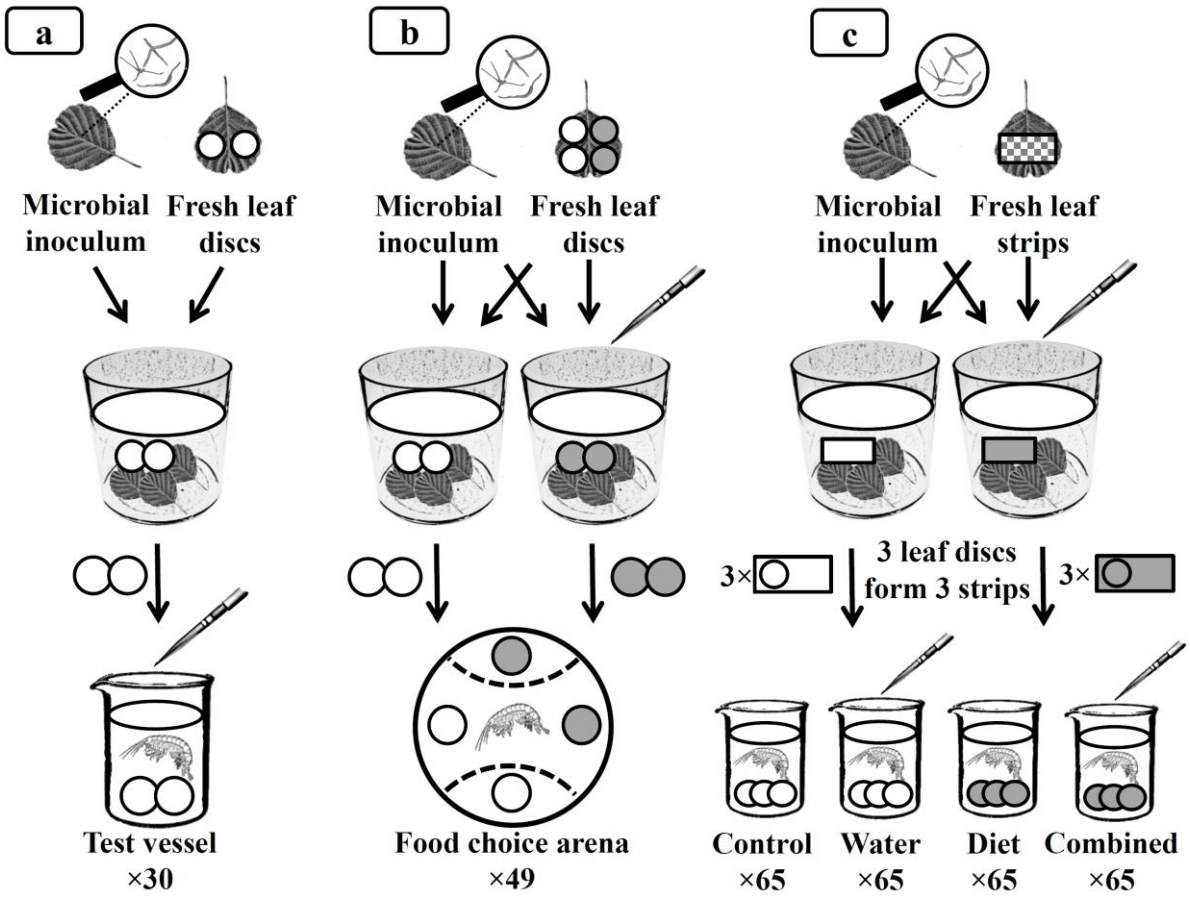

Fig. 3.1 Schematic overview of the three assay designs used for the assessment of waterborne and diet-related antimicrobial effects on the model decomposer-shredder system (a, b and c). Before the start of each assay, leaf discs and strips, respectively, cut from fresh black alder leaves were microbially conditioned (by using colonized leaves with a near-natural community, i.e., microbial inoculum) in the absence and presence (denoted by the pipette) of the antimicrobial(s). (a) shows the test design of the 7-day feeding activity assay where gammarids were exposed to the antimicrobial(s) via the water phase (denoted by the pipette). (b) displays the 24-hour food choice assay where the amphipod shredders were offered leaf discs microbially conditioned in the absence or presence of the antimicrobial(s) (denoted by white and grey discs, respectively). (c) displays the full-factorial study design of the 24-day long-term assay where the first factor was the absence or presence of the antimicrobial(s) in the water phase (denoted by the absence or presence of the pipette). The second factor constituted leaf discs as food source for the gammarids, which were microbially conditioned in the absence or presence of the antimicrobial(s) (denoted by white and grey discs, respectively; **Appendix A1 – A3**).

3.1.2 Diet-related fungicide effects in shredders

In the second part of phase one, fungicide-induced FQ-B effects on *G. fossarum* were investigated. Since sufficient data were available regarding waterborne toxicity of AZO to gammarids and AZO-induced changes in leaf-associated microbial communities (Zubrod et al., 2014; Zubrod et al., 2015a), a feeding activity and food choice assay for AZO were not

performed before conducting a 24-day long-term assay according to Zubrod et al. (2015b) (**Appendix A2**). However, due to unexpectedly high waterborne toxicity during a first long-term assay with 30 µg AZO/L, a follow-up assay with 15 µg AZO/L was carried out (**Appendix A2**). During both assays, the same response variables for *G. fossarum* as well as fungal biomass and bacterial density were determined as described for the long-term assay with CIP (3.1.1 Diet-related antibiotic effects in shredders). Additionally, amino acids (AA) of gammarids exposed to 30 µg AZO/L during the long-term assay were determined to explain unexpected waterborne AZO effects on somatic growth and energy reserves in gammarids. For this, gammarids' proteins were hydrolyzed for 24 h to free AAs via HCl (6 N) at 110 °C and AAs were quantified using an AA analysis Kit for GC-FID (cf. Badawy, 2019). Furthermore, instead of determining the NFLAs of microbially conditioned black alder leaves (see 3.1.1), their total FAs were analyzed to encompass all FAs potentially available to the amphipod shredder's diet. Therefore, FAs were transesterified and extracted via liquid-liquid extraction according Fink (2013). Afterwards, total FAs were measured and quantified as described above. For a detailed explanation of the experimental setup and all analyzes, see **Appendix A2**.

It was hypothesized that AZO, below concentrations causing waterborne effects in *G. fossarum*, negatively affects leaf-associated aquatic fungi. This, in turn, hampers microbial leaf conditioning, and thus reduces the nutritional quality of leaves for the amphipod shredder, ultimately leading to alterations in the gammarids' food assimilation (Zubrod et al., 2015c; Feckler et al., 2016). Besides potential dietary exposure effects due to fungicides that adsorbed to the leaves, the authors of these studies suggested that fungicides induced alterations in the leaf-associated fungal community in favor of less nutritional aquatic fungi reducing the quality of leaves, ultimately affecting the food assimilation and, thus, somatic growth of shredders.

3.1.3 Assessment and predictions of antimicrobial mixture effects

The last part of phase one aimed at assessing combined effects of antimicrobials in decomposer-shredder systems by subjecting the model shredder *G. fossarum* to the model antibiotic (CIP) and fungicide (AZO) via both effect pathways. Therefore, the same three-tiered testing approach was applied as described in 3.1.1. In the next step, observations were compared to effect predictions via the independent action (IA) model for dissimilar modes of action (Bliss, 1939). In order to realize the latter, data were used from this thesis

(**Appendix A1, A2**) as well as from previous publications (Zubrod et al., 2014; Zubrod et al., 2015a).

Table 3.1 Assays, source of experimental setups, as well as nominal concentrations of the mixture components and the binary antimicrobial mixture for each assay during phase 2 (**Appendix A3**).

Assay	Source of experimental setups	Mixture component	Nominal test concentration(s)	Mixture (AZO + CIP)	SI units
Feeding activity	Zubrod et al. (2014)	AZO	10.0; 27.5; 45.0; 62.5; 80.0	10.0 + 500; 27.5 + 500; 45.0 + 500; 62.5 + 500; 80.0 + 500	µg/L
	Appendix A1	CIP	500.0		µg/L
Food choice	Zubrod et al. (2015a)	AZO	0.1; 2.5	0.1 + 0.1; 0.1 + 2.5; 2.5 + 0.1; 2.5 + 2.5	mg/L
	Appendix A1	CIP	0.1; 2.5		mg/L
Long-term	**Appendix A2**	AZO	15.0	15.0 + 500.0	µg/L
	Appendix A1	CIP	500.0		µg/L

As AZO constitutes the toxic driver in the binary antimicrobial mixture due to its high toxicity towards aquatic invertebrates (European Food Safety Authority, 2010), five AZO concentrations combined with one fixed CIP concentration (Table 3.1) as well as a control (i.e., 0.0 + 0.0 µg/L AZO + CIP) were selected for the feeding activity assay (**Appendix A3**). Concentrations were below or equal to the effect concentration reducing the leaf consumption of gammarids by 20 % (EC_{20}) when exposed individually, since the EC_{20} is considered as an ecotoxicologically relevant benchmark that ensures a sufficient protection for aquatic organisms (Barnthouse et al., 2008). For the food choice assay, a factorial design was used with two concentrations each of AZO and CIP that were tested in all possible combinations (Table 3.1) and compared with the respective control (**Appendix A3**). The concentrations of each antimicrobial comprised the lowest observed effect concentration for microbial decomposition and a concentration inducing considerable effects on leaf-associated microorganisms (**Appendix A1**, Zubrod et al., 2015a). Finally, a 24-day long-term assay was performed with AZO and CIP concentrations, resulting in sublethal diet-related effects in gammarids when applied individually (Table 3.1; **Appendix A3**).

It was hypothesized that IA model predictions match observed effects in *G. fossarum* when induced by waterborne and dietary antimicrobial exposure, since the IA model by Bliss (1939) aims at predicting direct toxic effects of mixtures containing substances with dissimilar modes of action. However, effect predictions were not expected to comply with

observed FQ-B effects in gammarids, since the IA model is not designed to take complex horizontal and vertical species interactions into account.

3.2 Phase 2: diet-related algaecide effects in grazers

In phase two, a microcosm study was established to assess diet-related DIU effects on the consumption, somatic growth and NLFA profile of *P. acuta* fed with periphyton colonized in the absence or presence of DIU. A DIU concentration of 8 µg/L was selected, since clear effects on periphyton were anticipated (Piscart et al., 2009; Magnusson et al., 2012), while effects on the gastropod grazer via waterborne exposure were considered unlikely (López-Doval et al., 2014; **Appendix 4**). Ceramic tiles were colonized with periphyton for 21 days (6-day acclimatization phase without DIU followed by a 15-day expose phase) in stainless-steel channels of the Laboratory Stream Microcosm Facility at the iES Landau (Fig. 3.2a,b). The fully and homogeneously periphyton-covered tiles were subsequently used as food source for *P. acuta* during a 21-day long-term assay (Fig. 3.2c). To guarantee *ad libitum* feeding with periphyton during the entire long-term assay, three independent colonization phases were performed (Fig. 3.2d). To uncover mechanisms causing potential alterations in the food quality for *P. acuta*, biomass, cell viability, community structure and FAs of periphyton were determined. Total and autotrophic biomasses of periphyton were analyzed via ash free dry mass (AFDM) and content of chlorophylls (Chls) *a*, *b* and *c*, according to Biggs and Kilroy (2000) and Feckler et al. (2018), respectively. The phenotype-based algae cell composition (as proxy for DIU-induced shifts in the algae community structure; Sgier et al., 2018) and cell viability were characterized via flow cytometry and visual stochastic network embedding analysis, respectively (cf. Sgier et al., 2016). Moreover, the community composition of diatoms was analyzed as diatoms are often the predominant algae group in freshwater periphyton (Wu, 2017). This was performed by using DNA metabarcoding and high-throughput sequencing according to Mortágua et al. (2019). FAs of periphyton as proxy for food quality were quantified via GC FID as described in 3.1.1. For a detailed explanation of the experimental setup and all measured response variables, see **Appendix A4**.

It was hypothesized that DIU exerts a selection pressure within the periphyton community during the colonization phase causing alterations in its composition (Ricart et al., 2009). These changes in community composition would result in an altered nutritional quality of periphyton for the gastropod grazer, and, consequently, affect its feeding activity and physiological fitness (similar to observed fungicide effects in heterotrophic processes; cf. Zubrod et al., 2015c).

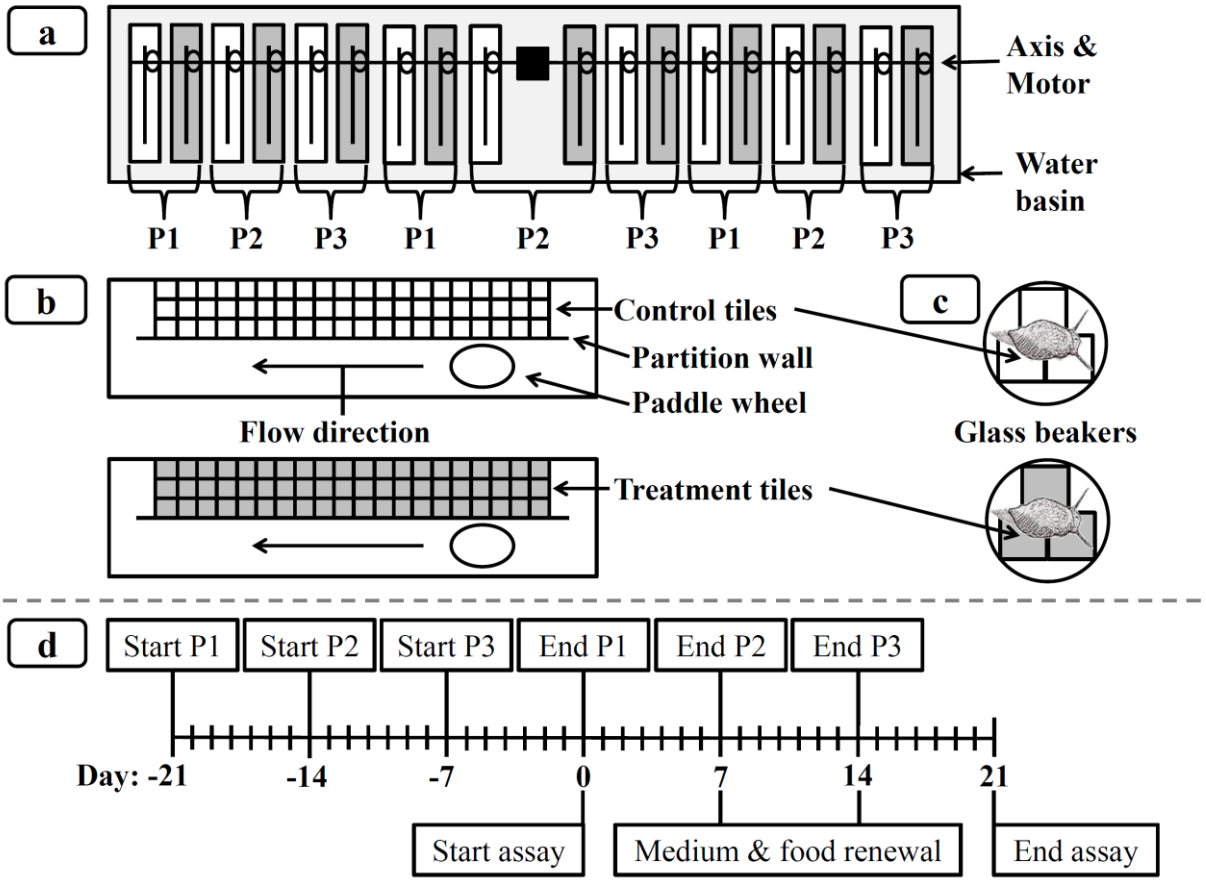

Fig. 3.2 Schematic overview of the microcosm study used for the assessment of diet-related antimicrobial effects on the model periphyton-grazer system. (a) displays the Landau Laboratory Stream Microcosm Facility where white and grey channels represent the control and the DIU treatment, respectively, and P1 – 3 are the three independent periphyton colonization phases partly running in parallel. (b) illustrates the setup of the experimental stream channels and (c) schematizes the replicates of the 21-day long-term assay with *P. acuta*. (d) constitutes the timeline of events in the periphyton colonization and the long-term assay (**Appendix A4**).

4 Results

4.1 Phase 1: diet-related antimicrobial effects in decomposer-shredder systems

4.1.1 Diet-related antibiotic effects in shredders

During the feeding activity assay, a statistically significant reduction in survival and leaf consumption of *G. fossarum* was observed at CIP concentrations in the mg/L range (Fig. 4.1; **Appendix A1**). The LC_{20} and LC_{50} values were 9.5 and 13.6 mg CIP/L and EC_{20} and EC_{50} values for the leaf consumption of gammarids were 0.5 and 6.4 mg CIP/L (Fig. 4.1; **Appendix A1**).

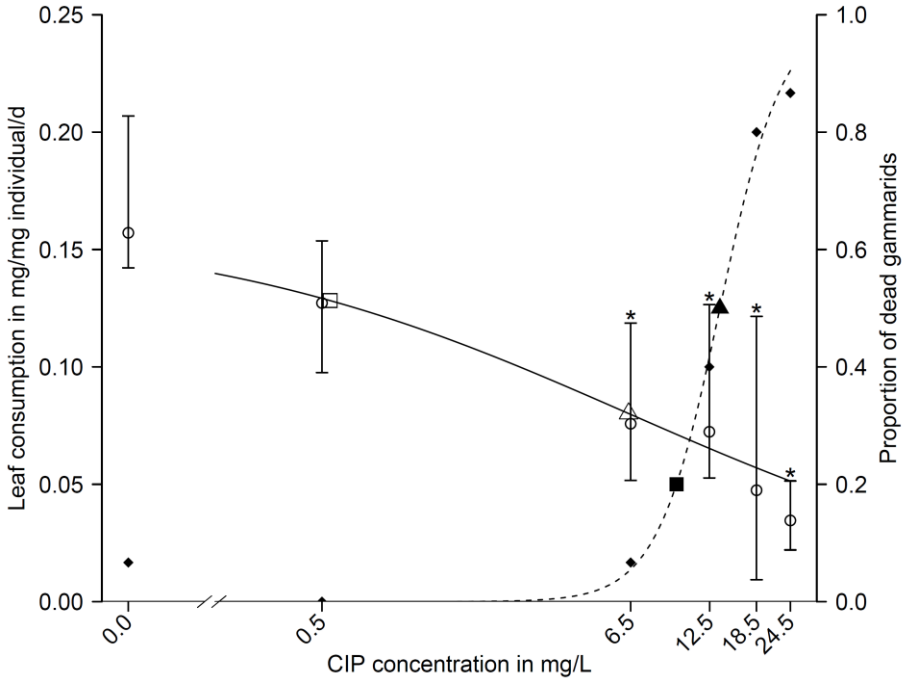

Fig. 4.1 Median leaf consumption (open circles with 95 % CIs) of *G. fossarum* and proportion of dead gammarids (solid diamonds) when subjected to increasing CIP concentrations. Moreover, the models with the best fit (solid line for leaf consumption and dashed line for mortality) as well as the EC_{20}/LC_{20} (transparent and solid squares, respectively) and EC_{50}/LC_{50} values (transparent and solid triangles, respectively) are displayed. Asterisks indicate a statistically significant difference to the control. Detailed results of statistical analyses can be found in **Appendix A1**.

During the food choice assay, ergosterol (as proxy for leaf-associated fungal biomass) was statistically significantly reduced by ~55 % and ~60 % at 500 and 2500 µg CIP/L, respectively, while no effects were observed for bacterial densities (**Appendix A1**).

Furthermore, the AH community composition was statistically significantly changed at 100 µg CIP/L (Fig. 4.2), which was mainly driven by negative CIP effects on the most dominant species *Fusarium* sp. (**Appendix A1**). *G. fossarum* did not show any statistically significant selective feeding behavior (Fig. 4.3; **Appendix A1**).

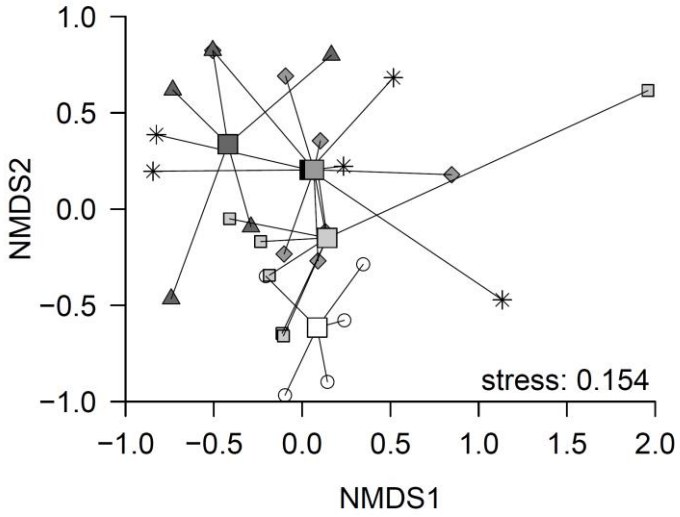

Fig. 4.2 Non-metric multidimensional scaling (NMDS) plot for hyphomycete communities associated with leaf material conditioned under control conditions (white circles) and in the presence of 20 (light grey squares), 100 (grey diamonds), 500 (dark grey triangles) and 2,500 (black asterisks) µg CIP/L during the food choice assay. A stress value is provided as a measure of "goodness-of-fit" for NMDS with reasonable fits indicated when below 0.2 (Clarke, 1993; detailed results of statistical analyses can be found in **Appendix A1**).

During the 24-day long-term assay, the leaf consumption and somatic growth of gammarids fed with leaf material exposed to CIP during the microbial conditioning were statistically significantly and non-significantly increased (~20 % and ~50 %, respectively). No effects for these response variables were observed when gammarids were subjected to waterborne CIP exposure (Fig. 4.4; **Appendix A1**). Furthermore, the saturated FA content and the NLFA composition of gammarids were statistically significantly enhanced (~30 %) and changed, respectively, via the dietary pathway (**Appendix A1**). Moreover, during the microbial conditioning of leaves used for long-term assay, fungal biomass was statistically significantly increased by ~180 %, while bacterial density was statistically non-significantly decreased (~25 %) when exposed to 500 µg CIP/L (**Appendix A1**). However, NLFA profiles of the leaf material microbially conditioned in the presence of CIP were not altered (**Appendix A1**).

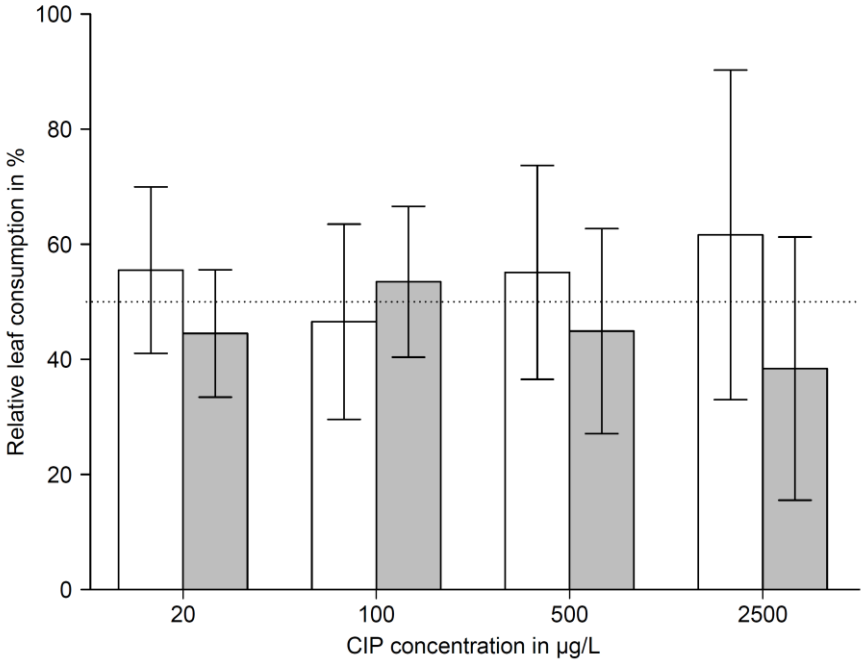

Fig. 4.3 Mean relative leaf consumption (with 95 % CIs) of *G. fossarum* on leaves microbially colonized under control conditions (white bars) or in the presence of increasing CIP concentrations (grey bars). The dotted line indicates the no-effect level (i.e., 50 % consumption on both leaf types; detailed results of statistical analyses can be found in **Appendix A1**).

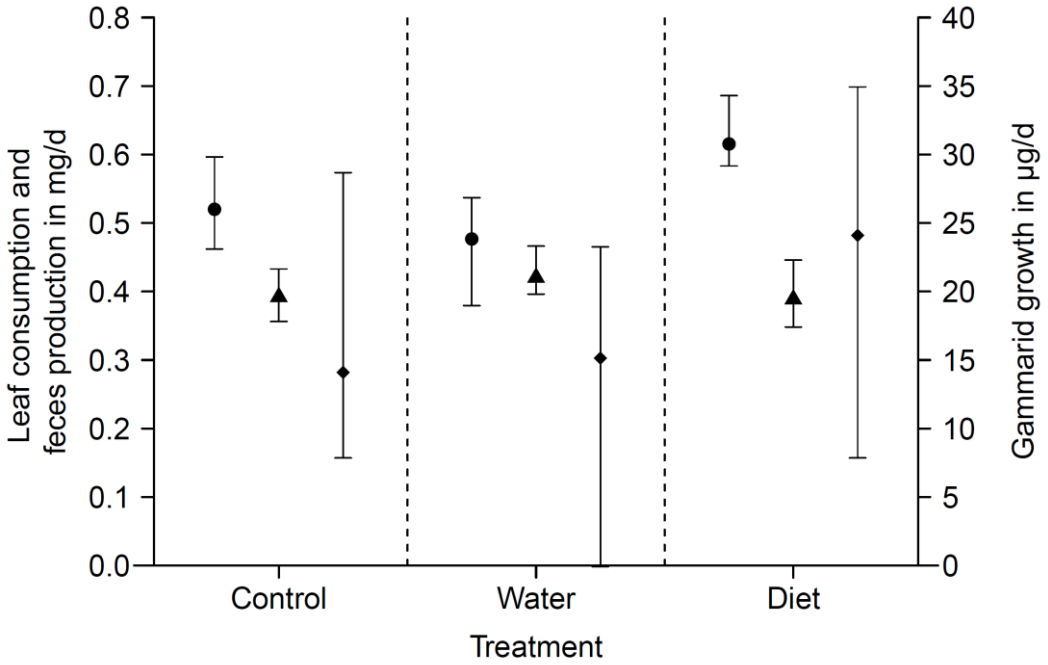

Fig. 4.4 Median (with 95 % CIs) leaf consumption (points), feces production (triangles) and growth (diamonds) of *G. fossarum* when gammarids were subjected to the waterborne (Water) and dietary effect pathway (Diet) during the 24-day long-term assay with CIP. Detailed results of statistical analyses can be found in **Appendix A1**.

4.1.2 Diet-related fungicide effects in shredders

In the first assay with 30 µg AZO/L, leaf consumption, feces production, somatic growth and survival of *G. fossarum* were statistically significantly reduced via waterborne exposure (Fig. 4.5, Fig. 4.6; **Appendix A2**), whereas NLFAs and AAs were statistically significantly and non-significantly increased, respectively (**Appendix A2**). Moreover, the AA composition of gammarids was statistically significantly changed, while their NLFA profiles were not altered (**Appendix A2**).

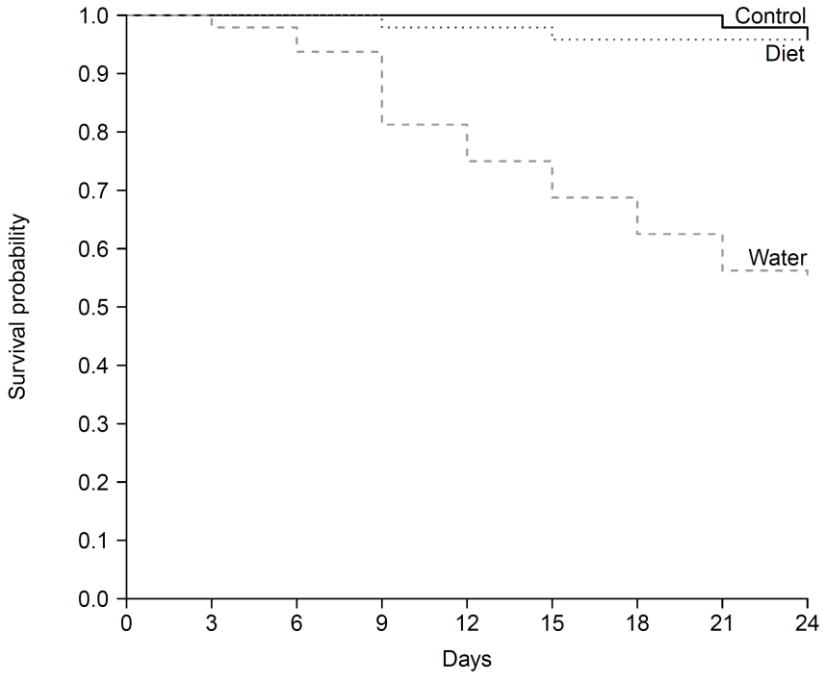

Fig. 4.5 Kaplan-Meier survival plot for *G. fossarum* subjected to the Control (black solid line), Water (light gray dashed line) and Diet (dark gray dotted line) treatment during the 24-day bioassay with 30 µg AZO/L. Detailed results of statistical analyses can be found in **Appendix A2**.

Due to the high waterborne toxicity, a second long-term assay with 15 µg AZO/L was performed, where no waterborne effects on gammarids were observed (**Appendix A2**). During this assay, energy processing of gammarids via the dietary pathway was statistically significantly changed (i.e., increased feces production with constant leaf consumption, Fig. 4.6; **Appendix A2**), whereas no effects were observed on the other measured response variables (**Appendix A2**). The total FA content and composition of leaf material microbially conditioned in the presence of 15 µg AZO/L were statistically significantly enhanced and altered, respectively (Table 4.1; **Appendix A2**). No effects were observed for fungal biomass and bacterial density (Table 4.1; **Appendix A2**).

Table 4.1 Number of analyzed replicates and group medians (with 95 % CIs) of parameters describing leaf quality used during the 24-day bioassays. Additionally, the statistical tests used for the respective endpoint as well as the *p*-value from the statistical comparison of 30 or 15 µg AZO/L with the control is shown. All *p*-values below 0.05 are printed in bold (**Appendix A2**).

Endpoint	Concentration (µg/L)	*n*	Median	±95 % CI	Statistical test	*p*-value
Fungal biomass in µg ergosterol/g leaf dry mass	0	8	29.38	8.16 to 54.16	Student's *t*	
	30	8	38.78	11.58 to 89.41		0.128
Bacterial density in 10^9 cells/g leaf dry mass	0	8	0.67	0.24 to 2.44	Wilcoxon rank-sum	
	30	8	0.77	0.28 to 2.08		0.645
Fungal biomass in µg ergosterol/g leaf dry mass	0	12	61.04	42.49 to 107.72	Student's *t*	
	15	11	64.53	17.56 to 107.83		0.830
Bacterial density in 10^9 cells/g leaf dry mass	0	12	0.59	0.41 to 0.83	Wilcoxon rank-sum	
	15	12	0.71	0.38 to 0.92		0.799
Total FA content in µg/mg leaf dry mass	0	12	10.76	9.95 to 13.94	Wilcoxon rank-sum test	
	15	12	15.02	11.19 to 16.97		**0.033**
Saturated FA content in µg/mg leaf dry mass	0	12	4.45	4.04 to 5.90	Wilcoxon rank-sum test	
	15	12	5.71	4.77 to 6.96		0.068
Monounsaturated FA content in µg/mg leaf dry mass	0	12	1.64	1.12 to 2.08	Student's *t*	
	15	12	2.12	1.72 to 2.36		**0.019**
Polyunsaturated FA content in µg/mg leaf dry mass	0	12	4.95	4.41 to 6.23	Wilcoxon rank-sum test	
	15	12	6.89	5.11 to 8.11		0.078
FA composition of leaves	0	12	-	-	PERMANOVA	
	15	12	-	-		**0.023**

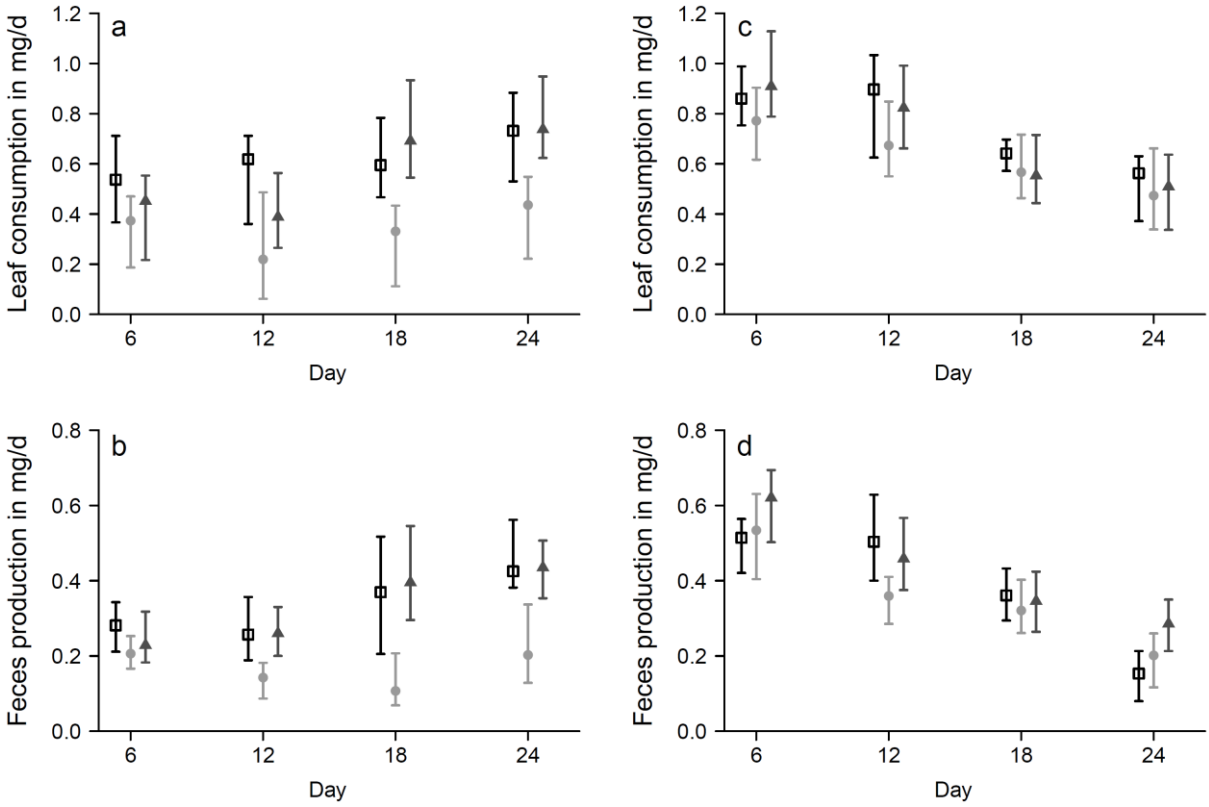

Fig. 4.6 Median (with 95 % CIs) leaf consumption and feces production of *G. fossarum* subjected to the Control (black square), Water (light gray dot) and Diet (dark gray triangle) treatments during the 24-day bioassays with 30 (a and b) and 15 (c and d) µg AZO/L. Detailed results of statistical analyses can be found in **Appendix A2**.

4.1.3 Assessment and predictions of antimicrobial mixture effects

Generally, mixture effect predictions of the IA model matched the observed waterborne effects on gammarids' leaf consumption (i.e., predictions were within the 95 % confidence intervals (CIs) of mean observed effects) during the 7-day feeding activity assay, except for the lowest test concentration (Fig. 4.7; **Appendix A3**).

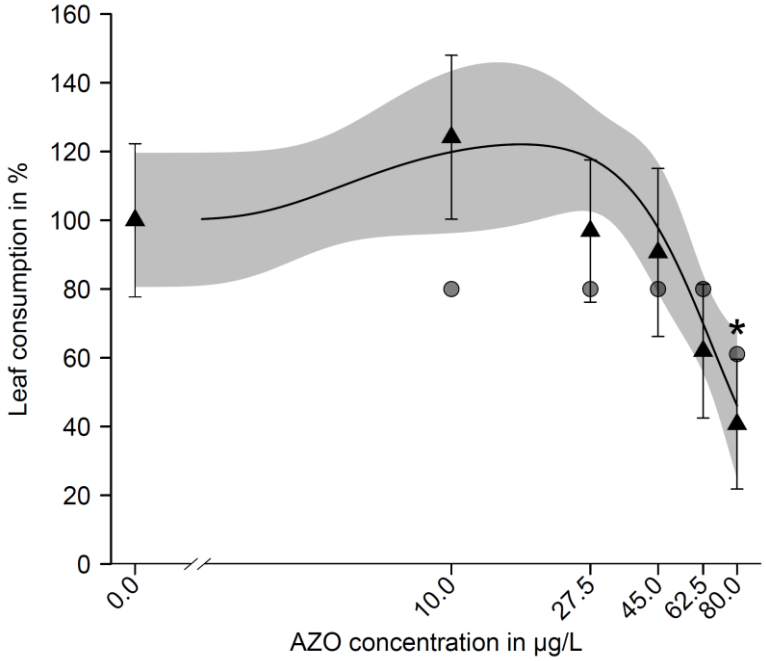

Fig. 4.7 Mean (with 95 % CIs) percentage effect on the leaf consumption of gammarids (black triangles) when the animals were subjected to the binary antimicrobial mixture with increasing AZO concentrations and a fixed CIP concentration of 500 µg/L. Moreover, the model with the best fit (black line with 95 % CIs) and IA predictions (grey circles) derived from the feeding activity assays, where the mixture components were tested individually, are displayed. The asterisk indicates a statistically significant difference to the control. Detailed results of statistical analyses can be found in **Appendix A3**.

IA predictions partly matched observed antimicrobial mixture effects on microbial leaf decomposition during the food choice assay, whereas most effect predictions fell within the 95 % CIs of the median observed food selection of *G. fossarum*, except for one treatment (Fig. 4.8; **Appendix A3**). Additionally, the antimicrobial mixtures statistically significantly reduced fungal biomass, bacterial density and fungal diversity (indicated by sporulation of AH; **Appendix A3**).

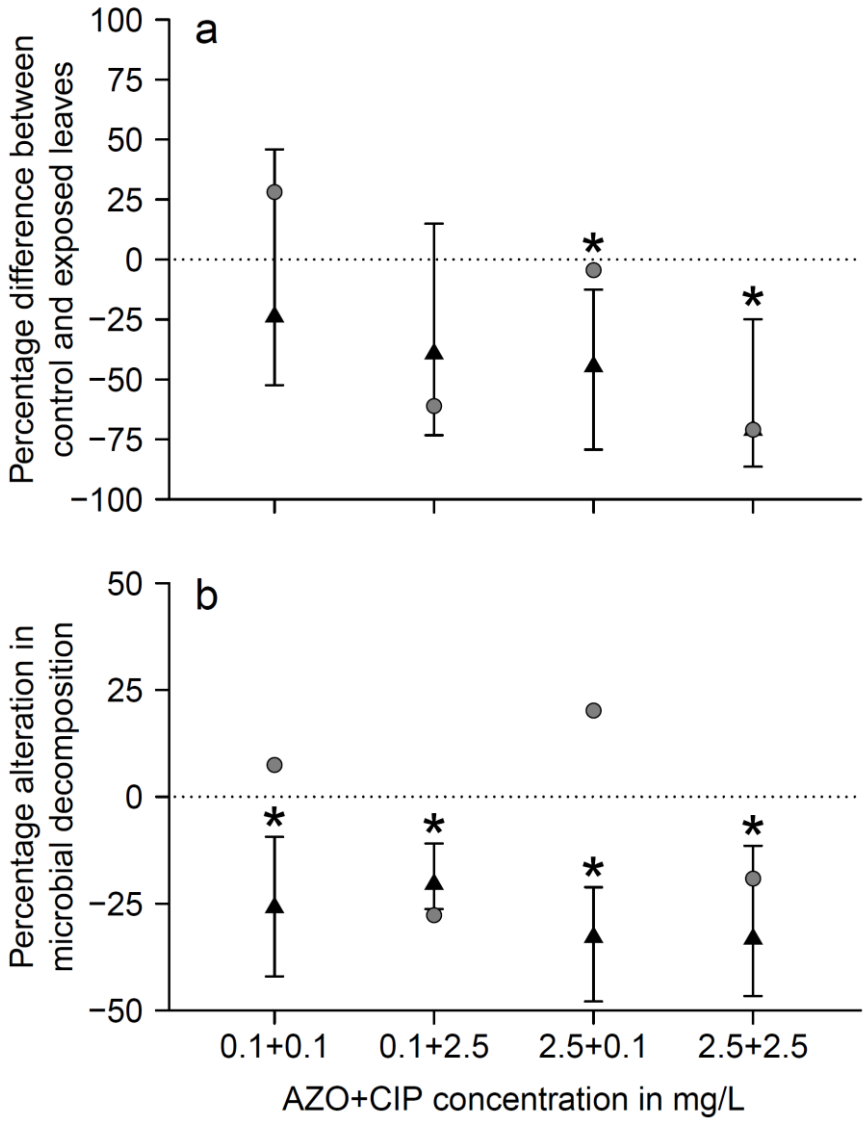

Fig. 4.8 Median (with 95 % CIs) percentage effect (relative to the respective control) of (a) food selection of gammarids and (b) microbial leaf decomposition (black triangles) when subjected to different concentrations of the binary antimicrobial mixture. Furthermore, IA predictions derived from the food choice assays with the individual tested mixture components are displayed as grey circles. Asterisks indicate a statistically significant difference to the control. Detailed results of statistical analyses can be found in **Appendix A3**.

As expected, during the 24-day long-term assay, no waterborne toxicity of the antimicrobial mixture to gammarids was observed, while all measured response variables were statistically significantly or non-significantly increased when *G. fossarum* was subjected to the dietary effect pathway (**Appendix A3**). For the latter pathway, effect predictions for leaf consumption and somatic growth of gammarids matched the observed effects, whereas the IA model prediction for feces production was not within the 95 % CI of the median observed effect (Fig. 4.9 **Appendix A3**). Contrary to the long-term assays conducted with the

individual mixture components (i.e., CIP and AZO; see above), leaf quality-related parameters (fungal biomass, AH community composition and FAs of conditioned leaf material) were not altered when leaf material was exposed to the antimicrobial mixture during the microbial conditioning (**Appendix A3**).

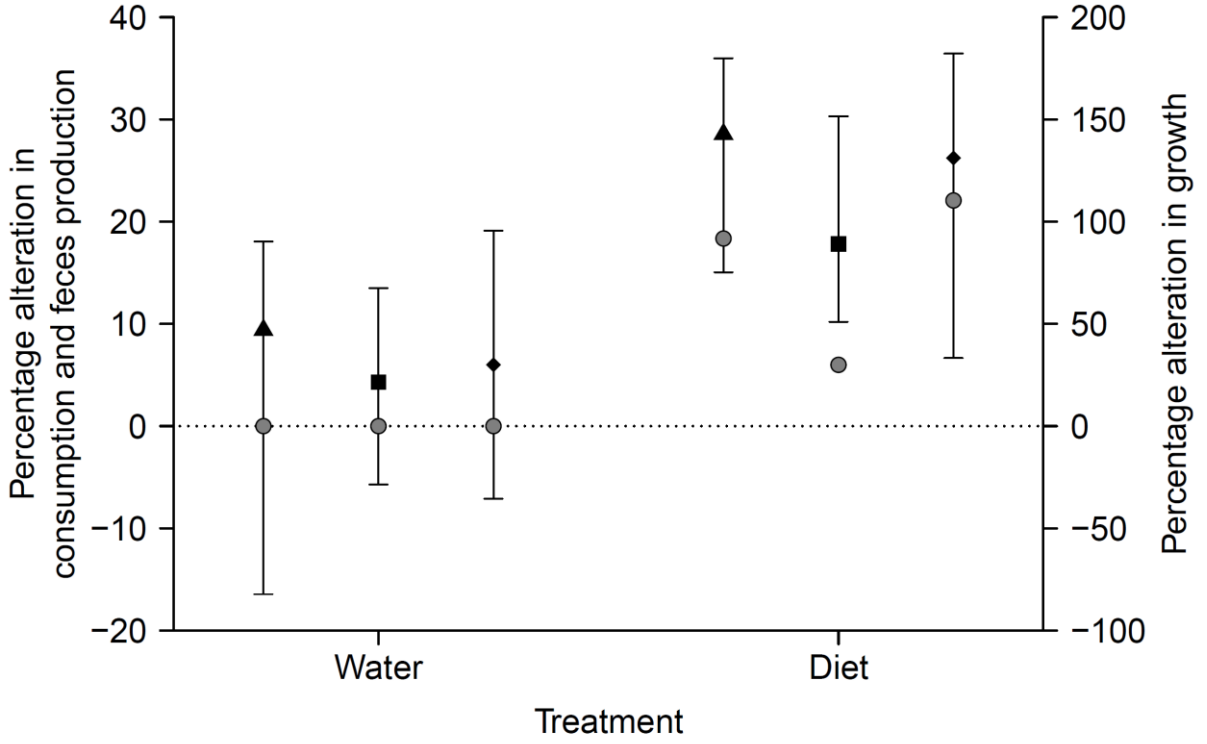

Fig. 4.9 Median (with 95 % CIs) percentage effect (relative to the control) on the leaf consumption (black triangles), feces production (black squares) and growth of *G. fossarum* (black diamonds) when gammarids were subjected to the waterborne (Water) and dietary effect pathway (Diet) during the 24-day long-term assay with binary antimicrobial mixtures. IA predictions derived from the long-term assays with the individual tested mixture components are illustrated as grey circles. Detailed results of statistical analyses can be found in **Appendix A3**.

4.2 Phase 2: diet-related algaecide effects in grazers

During the periphyton colonization, no statistically significant effects on periphyton biomass (i.e., AFDM and Chls) were observed when periphyton was exposed to 8 µg DIU/L (**Appendix A4**). However, DIU induced statistically significant changes in the phenotype-based algae cell composition (Fig. 4.10; **Appendix A4**), as well as the relative sequence abundances of diatoms (Fig. 4.11; **Appendix A4**), and increased the diatom diversity (at the operational taxonomic unit level; **Appendix A4**). Moreover, FA profiles of periphyton were statistically significantly altered in the presence of DIU tending to higher amounts of HUFAs (such as 20:5n-3 and 20:4n-6; Fig. 4.12; **Appendix A4**).

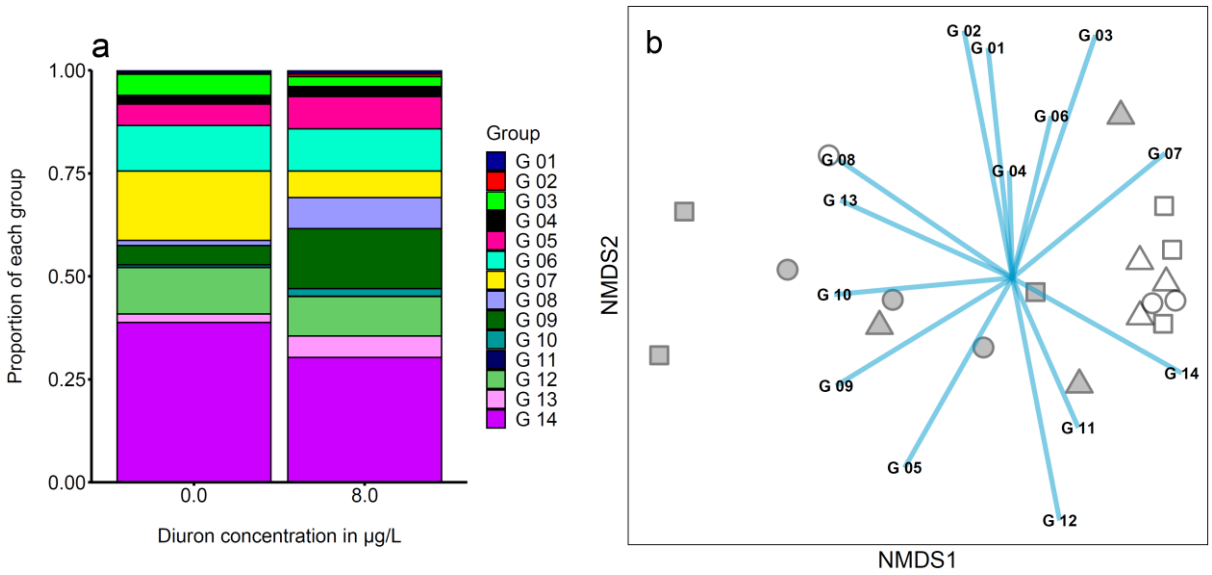

Fig. 4.10 (a) Mean relative abundance (0 – 1) and (b) NMDS plots for phenotype-based cell groups (G 01 – G 14) exposed to 0 (i.e., control; white symbols) and 8 µg DIU/L (grey symbols) during the periphyton colonization. Circles, squares and triangles constitute the first, second and third periphyton colonization phase, respectively. The stress value (as a measure of "goodness-of-fit") was below 0.2, indicating a reasonable fit (Clarke, 1993), and blue lines display the impact of each group. Detailed results of statistical analyses can be found in **Appendix A4**.

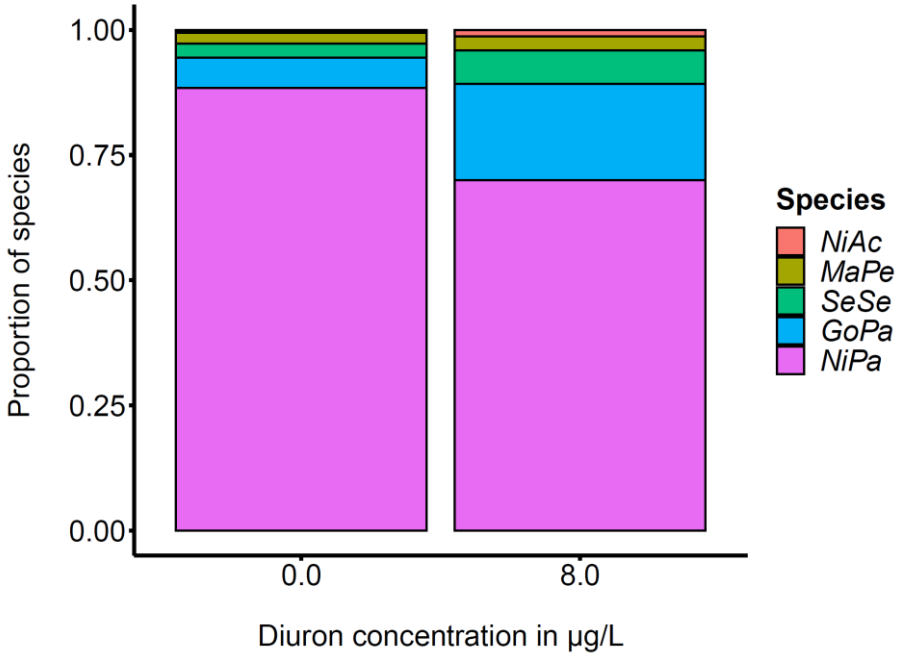

Fig. 4.11 Mean relative sequence abundance (0 – 1) of the five most prevalent diatom species NiAc (*Nitzschia acidoclinata*), MaPe (*Mayamaea permitis*), SeSe (*Sellaphora seminulum*), GoPa (*Gomphonema parvulum*) and NiPa (*Nitzschia palea*) exposed to 0 (i.e., control) and 8 µg DIU/L during the periphyton colonization. Detailed results of statistical analyses can be found in in **Appendix A4**.

When *P. acuta* was fed with periphyton that was exposed to DIU during the 21-day long-term assay, the snails' consumption was statistically significantly increased over time in comparison to the control (Fig. 4.13; **Appendix A4**). Moreover, NLFA content and profiles of snails were statistically significantly enhanced (~65 %) and altered (Fig. 4.12; **Appendix A4**), respectively, while somatic growth of *P. acuta* was statistically non-significantly increased by ~55 % compared to the control at test termination (**Appendix A4**).

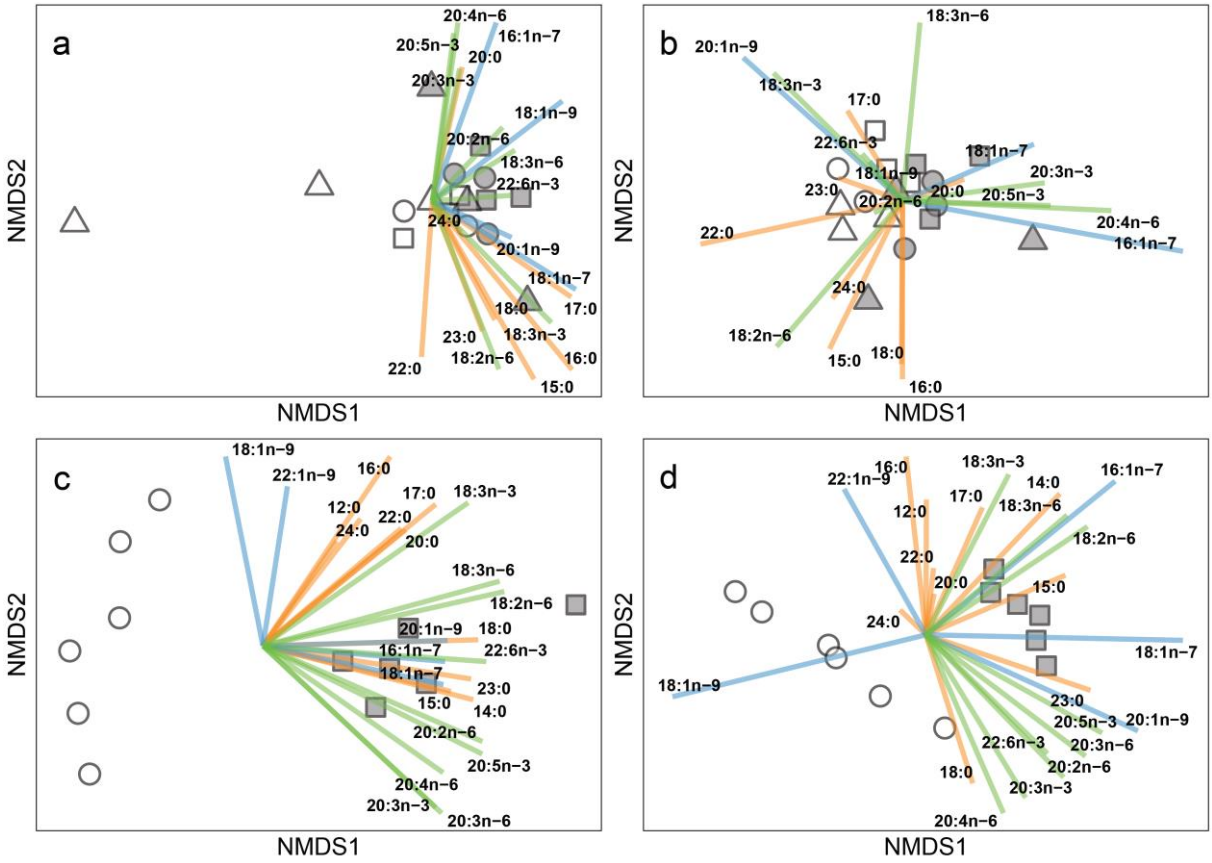

Fig. 4.12 NMDS plots for the FA composition of periphyton exposed to 0 (i.e., control; white symbols) and 8 µg DIU/L (grey symbols) during the periphyton colonization in (a) total amounts (in µg/mg) and (b) relative abundances (0 – 1). Circles, squares and triangles constitute the first, second and third periphyton colonization phase, respectively. In addition, the NLFA composition of snails fed with periphyton colonized under control conditions (white circles) and in the presence of 8 µg DIU/L (grey squares) during the 21-day long-term assay in (c) total amounts and (d) relative abundances. Stress values (as a measure of "goodness-of-fit") for all NMDS were below 0.2, indicating reasonable fits (Clarke, 1993). Orange (saturated FAs), blue (monounsaturated FAs) and green (polyunsaturated FAs) lines display the impact of each FA. Detailed results of statistical analyses can be found in **Appendix A4**.

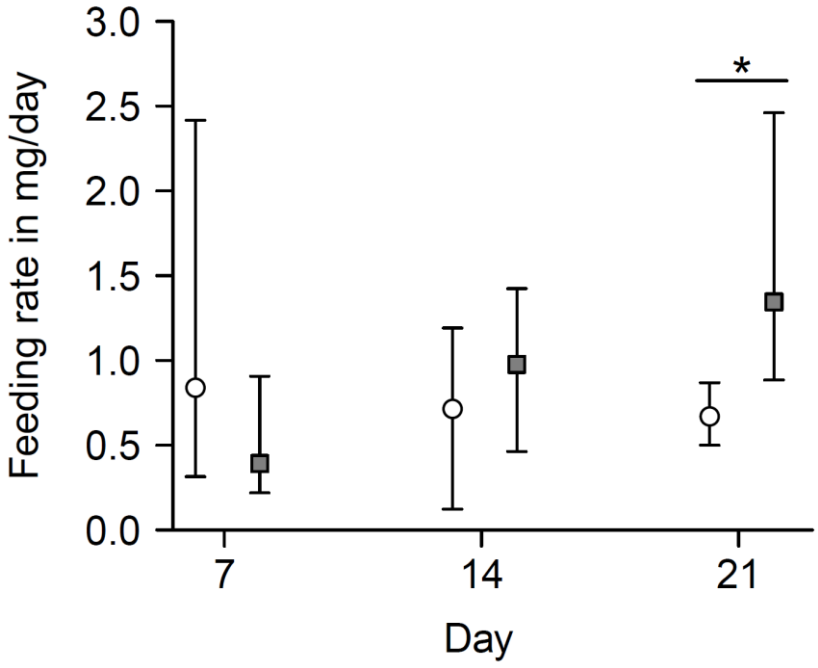

Fig. 4.13 Median (with 95 % CIs) consumption of *P. acuta* fed with periphyton colonized under control conditions (white circles) and in the presence of 8 µg DIU/L (grey squares) during the 21-day long-term assay. The asterisk denotes a statistically significant difference to the control. Detailed results of statistical analyses can be found in **Appendix A4**.

5 Discussion

5.1 Phase 1: diet-related antimicrobial effects in decomposer-shredder systems

5.1.1 Diet-related antibiotic effects in shredders

Using a feeding activity assay, it was demonstrated that *G. fossarum* is tolerant to waterborne CIP toxicity (**Appendix A1**), which is in agreement with reported results for other crustaceans (e.g., *Daphnia* spp.; Martins et al., 2012; Dalla Bona et al., 2014). As hypothesized, microbial decomposers were more sensitive than the amphipod shredder when exposed to CIP during the microbial conditioning of the food choice assay (**Appendix A1**). Unexpectedly, the antibiotic substantially reduced aquatic fungi biomass, while bacterial density remained unaffected (**Appendix A1**). This could be due to the fact that the prevalent hyphomycete species *Fusarium* sp. was negatively affected by the fluoroquinolone, resulting in a decreased competitive pressure for bacteria (**Appendix A1**). The reduced competitive pressure by aquatic fungi along with the rapid adaptation of bacteria to chemical stress (Brandt et al., 2015) probably led to no effects on bacteria density (**Appendix A1**). As aquatic fungi substantially increase the nutritional value of leaf litter (Bärlocher and Kendrick, 1973; Bärlocher and Kendrick, 1975), this shift in the microbial community may have affected the food quality of leaves for *G. fossarum*, although gammarids showed no statistically significant food selection during the food choice assay (**Appendix A1**).

Similar to the food choice assay, an altered food quality of leaves microbially conditioned in the presence of 500 µg CIP/L is also suggested during the long-term assay (indicated by an enhanced fungal biomass; Foucreau et al., 2013; **Appendix A1**). However, contrary to the outcome of the food choice assay (but in line with the hypothesis in 3.1.1), due to adverse effects of CIP to leaf-associated bacteria, aquatic fungi were probably released from competitive pressure by bacteria, resulting in an increased fungal growth. This assumption is – in addition to increased fungal biomass – also supported by a reduced bacterial density in the presence of CIP (**Appendix A1**). As expected, the increase in fungal biomass probably stimulated the leaf consumption of gammarids in the Diet treatment and ultimately resulted in an increased somatic growth and energy storage (**Appendix A1**). Moreover, the enhanced leaf consumption probably triggered a faster adaptation of the gammarids‘ NLFA profile to the diet that was provided during the assay compared to the test

organisms in the Control and Water treatment (**Appendix A1**). The contrasting results to the food choice assay can be explained by the absence of CIP-sensitive AHs (e.g., *Fusarium* sp.) during the long-term assay (**Appendix A1**). As the food choice and long-term assay were conducted at different seasons (August vs. January), seasonal variations in microbial communities are most likely responsible for different effects on leaf-associated microorganisms during both assays (Nikolcheva and Bärlocher, 2005). However, the increase in the leaf-associated fungal biomass during the long-term assay was not reflected in changes in the NLFA profile of the leaf material that was microbially conditioned in the presence of CIP during the long-term assay (**Appendix A1**). Possibly, aquatic fungi invested in somatic growth (increase in phospholipid FAs, structural cell membrane components; Willmer et al., 2005) instead of energy storage (NLFAs) due to the surplus of nutrients from leaves and the culture medium provided during the microbial conditioning (Bååth, 2003).

These data indicate that CIP probably altered the leaf-associated microbial community composition, which, in turn, induced FQ-B effects in *G. fossarum* (see RQ1 in 1.2). Nevertheless, it cannot be ruled out that dietary CIP exposure induced changes in the gut microbiome or the immune system of *G. fossarum* resulting in the observed effects (**Appendix A1**), since both have an impact on the behavior and energy processing of animals (Brown et al., 2017).

5.1.2 Diet-related fungicide effects in shredders

The first long-term assay showed that waterborne AZO exposure at 30 µg/L substantially reduced the feeding activity of *G. fossarum* (**Appendix A2**), most likely to cope with the chemical stress (Maltby, 1999). As a consequence of the reduced energy intake and probably combined with higher maintenance costs covering detoxification and damage repair due to cellular oxidative stress induced by AZO (Elskus, 2012), waterborne AZO toxicity caused energy trade-offs in terms of reduced growth (**Appendix A2**). Furthermore, the oxidative stress triggered by AZO exposure was likely responsible for the decreased survival of *G. fossarum* by damaging vital cellular biomolecules, which, in turn, resulted in cell death (Lushchak, 2011; **Appendix 2**). Moreover, since AZO is suggested as metabolic disruptor due to the impairment of mitochondrial respiration and thus disrupting the catabolism in organisms (Kassotis and Stapleton, 2019), NLFAs and AAs of gammarids probably could not be utilized for metabolic processes, and thus accumulated in the animals subjected to waterborne AZO exposure (**Appendix A2**). Changes in the AA composition were also observed during the assay (while NLFA composition was unaffected; **Appendix A2**) but

cannot be interpreted in this thesis, since biochemical processes behind pollutant-induced changes in the AA structure of invertebrates are poorly studied.

While diet-related AZO effects seemed to be of minor importance for *G. fossarum* compared to waterborne AZO toxicity during the 24-day long-term assay with 30 µg AZO/L, in the second assay with 15 µg AZO/L, the dietary pathway was more relevant (**Appendix A2**). The increased feces production at consistent leaf consumption indicates a lower food assimilation efficiency of gammarids, which was probably induced by alterations in the food quality of leaves that were microbially conditioned in the presence of AZO (**Appendix A2**). This is supported by the higher FA content of the conditioned leaf material compared to the control indicating a lower microbial activity (Torres-Ruiz and Wehr, 2010) resulting from direct or indirect (i.e., alterations in the community composition) AZO effects in the microbial decomposition efficiency (**Appendix A2**). However, since AZO-induced changes in food quality for *G. fossarum* were not reflected in the NLFA composition of gammarids, which would point to substantial shifts in the microbial community composition, direct fungicide effects in the microbial decomposition efficiency is more likely (**Appendix A2**).

As hypothesized (see 3.1.2), the data indicate FQ-B effects on gammarids at 15 µg AZO/L, which concurs with studies observing similar fungicide effects on crustacean shredders via the dietary pathway (Zubrod et al., 2015c; Feckler et al., 2016; RQ1 in 1.2). Since no harmful waterborne effects on gammarids were observed at 15 µg AZO/L, this concentration was selected for the assessment of antimicrobial mixture effects on the energy processing and physiology of *G. fossarum* by separating the waterborne and dietary effect pathway (**Appendix A3**).

5.1.3 Assessment and predictions of antimicrobial mixture effects

In the first step, waterborne antimicrobial mixture effects of AZO and CIP were assessed to check for unexpected synergistic effects on *G. fossarum* via this pathway. No synergistic interactions of the antimicrobial mixture on the leaf consumption of gammarids were observed when gammarids were exposed for 7 days at concentrations of AZO and CIP below or equal to the EC_{20} values of the individual mixture components (**Appendix A3**). Although the observation of a hormetic effect at the lowest mixture concentration indicates an increased energy demand due to the chemical stress, resulting in the higher energy intake (Eriksson-Wiklund et al., 2011), an increased risk for adverse effects in *G. fossarum* from combined waterborne exposure of AZO and CIP cannot be assumed (**Appendix A3**).

For microbial decomposers, synergism of antimicrobial mixtures was partly observed during the food choice assay when the individual components of the mixture triggered effects in opposite directions (i.e., 0.1 mg CIP/L and 2.5 mg AZO/L induced an increase and decrease, respectively, in microbial activity; **Appendix A3**). This was also reflected in the food selection of *G. fossarum*, although not as pronounced as in the microbial activity (**Appendix A3**). Additionally, the decrease in fungal biomass and diversity caused by AZO and CIP indicate a reduced food quality for shredders, suggesting potential FQ-B effects on the energy processing and physiology of *G. fossarum* when fed with exposed leaf material over a longer period (**Appendix A3**).

During the 24-day long-term assay, no synergistic interactions of AZO and CIP were observed when *G. fossarum* was subjected to the waterborne or dietary effect pathway, except for the feces production of gammarids in the Diet treatment. This might be explained by the increased median leaf consumption of *G. fossarum* that deviated by 10 % from the prediction of the IA model (**Appendix A3**). The stimulated food intake, which ultimately led to an increased somatic growth and energy reserves, was probably not induced by microbial-mediated changes in the food quality for gammarids, as no changes were observed in the microbial community structure of leaf material conditioned in the presence of the antimicrobial mixture (**Appendix A3**). It thus seems more likely that dietary CIP exposure caused alterations in the immune system or gut microbiome of gammarids resulting in the observed effects (see also 5.1.1 for CIP exposure above; **Appendix A3**).

The results in the last part of phase one indicate that IA model predictions for direct effects of AZO and CIP in gammarids (i.e. via waterborne and dietary exposure) are accurate. However, as hypothesized (see 3.1.3), indirect mixture effects on *G. fossarum* are hard to predict, since model predictions are inaccurate for effects on microbial decomposer communities due to complex horizontal interactions between bacteria and aquatic fungi (Romaní et al., 2006; **Appendix A3**). Therefore, according to RQ2 (see 1.2), diet-related antimicrobial mixture effects can only be partly predicted in freshwater biofilm-consumer systems (**Appendix A3**).

5.2 Phase 2: diet-related algaecide effects in grazers

As expected, changes in the phenotype-based algae cell composition and relative abundances of diatoms in the presence of DIU indicate algaecide-induced alterations in the periphyton community structure during the periphyton colonization (**Appendix A4**). It is likely that DIU-sensitive algae species, such as *N. palea* (**Appendix A4**), were replaced by more tolerant species (e.g., *Gomphonema parvulum*; Larras et al., 2014) within the community due to a competitive disadvantage under algaecide stress (Schmitt-Jansen and Altenburger, 2005b). These changes in community composition are most likely responsible for alterations in the periphyton FA profiles (**Appendix A4**), since FAs can vary between different algae (e.g., Taipale et al., 2013). The increase in HUFAs (a proxy for high-food quality; Brett and Müller-Navarra, 1997) in periphyton exposed to DIU, probably stimulated the periphyton consumption of *P. acuta*, which, in turn, enhanced the energy reserves (i.e., NLFAs) of the snails as well as their somatic growth by trend (**Appendix A4**). Moreover, the enhanced consumption induced a faster shift in the NLFA profile of *P. acuta* to higher concentrations of HUFAs suggesting that the physiological fitness of snails was higher in the DIU treatment than in the control at termination of the assay (**Appendix A4**).

In contrast, an increased periphyton consumption of *P. acuta* to compensate food with low quality is also possible (Fink and Elert, 2006). This seems, however, unlikely, since compensatory feeding is associated with lower somatic growth of the animal due to enhanced energy expenditures for increased activity (e.g., Plath and Boersma, 2001; Fink and Elert, 2006), which was not observed in this thesis (**Appendix A4**). Furthermore, the total amount of HUFAs of periphyton was comparable or higher than the control (**Appendix A4**), suggesting no reduced food quality for the snails in the DIU treatment. Another potential explanation for the observed effects in *P. acuta* may be direct DIU effects due to dietary exposure, although the concentration of 8 µg/L used in the present study is comparatively low for the grazer. During a preliminary test, in which waterborne DIU toxicity to *P. acuta* was investigated using a 7-day feeding activity assay, the leaf consumption of snails was not affected at nominal concentrations of up to 50 mg/L (**Appendix A4**). Considering that no effects were observed at the mg/L-range during this study and the estimated median intake of DIU by *P. acuta* was ~12 µg during the entire long-term assay (**Appendix A4**), effects on the snails' periphyton consumption by dietary exposure seem unlikely, despite differences in the study durations. Therefore, it is suggested that FQ-B effects induced by DIU positively affected *P. acuta* during the long-term assay (see RQ3 in 1.2; **Appendix A4**).

6 Conclusion and future perspectives

This thesis demonstrates the relevance of diet-related effects of antimicrobials and their mixtures on shredders and grazers as important effect pathway. The results show not only the potential impact of antimicrobials on consumers via dietary exposure (as indicated for CIP; **Appendix A1, A3**) but also highlight the importance of FQ-B effects in freshwater biofilm-consumer systems at environmentally relevant concentrations (**Appendix A2, A4**). These findings are informative for government agencies that are involved in the authorization of pesticides, biocides or pharmaceuticals, since indirect effects as well as effects of mixtures across chemical classes and regulatory sectors (e.g., mixtures of pesticides and pharmaceuticals) are currently ignored in the ERA of these chemical groups (Bopp et al., 2019; Topping et al., 2020). In particular, FQ-B effects could be highly relevant, because they may have far-reaching implications for entire food webs (**Appendix A4**): for instance, alterations in the community composition of biofilms can change the availability of essential nutrients for consumers such as merolimnic insects, which, in turn, can affect the food quality for higher trophic levels in aquatic ecosystems that use these insects as food source (Thera et al., 2020). Moreover, these effects could propagate across ecosystem boundaries, as merolimnic aquatic animals constitute a crucial diet for consumers in terrestrial ecosystems (Martin-Creuzburg et al., 2017), suggesting consequences for aquatic-terrestrial meta-ecosystems (Schulz et al., 2015). Therefore, future studies should address this issue by incorporating higher trophic levels to investigate antimicrobial-induced cascading effects in food webs.

Despite the strong indications in the present thesis that antimicrobials induced FQ-B effects on *G. fossarum* and *P. acuta*, it cannot be ruled out that dietary exposure was involved in the effects observed in the consumers. Therefore, future research should aim to separate direct and indirect antimicrobial effects via the dietary effect pathway. This could, for instance, be achieved by using factorial test designs with artificial microbial communities. The first factor could constitute consumers being fed with a substrate-associated artificial community colonized in the absence or presence of an antimicrobial. The second factor would then comprise consumers being fed with an artificial community that has been modified to constitute the post-exposure community, with or without spiking the antimicrobials under investigation. Although such study designs are technically challenging, for example, due to the required modifications of microbial communities, first steps in this direction have already been taken. For instance, Baudy et al. (2021) investigated fungicide-induced alterations in an

artificial AH community, which revealed modifications of fungal communities in presences of fungicides. In addition, comprehensive analyses of the community composition (e.g., via metagenomics) and microorganism-associated micro- and macro-nutrients (e.g., inter alia nutrient stoichiometry, FA and AA analysis), as well as the determination of internal antimicrobial concentrations and stress biomarkers (e.g., glutathione S-transferase) in the consumer would help to facilitate the understanding of indirect and direct effects (**Appendix A4**). Those comprehensive investigations would help to understand the underlying mechanisms of diet-related effects in consumers and fill knowledge gaps identified in the present thesis.

7 References

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Appendix

Appendix A1: Konschak, M., Zubrod, J.P., Baudy, P., Fink, P., Kenngott, K.G.J., Lüderwald, S., Englert, K., Jusi, C., Schulz, R., Bundschuh, M., 2020. The importance of diet-related effects of the antibiotic ciprofloxacin on the leaf-shredding invertebrate *Gammarus fossarum* (Crustacea; Amphipoda). Aquat. Toxicol. 222, 1–10.

Appendix A2: Konschak, M., Zubrod, J.P., Baudy, P., Kenngott, K.G.J., Englert, D., Röder, N., Ogbeide, C., Schulz, R., Bundschuh, M., 2021. Chronic effects of the strobilurin fungicide azoxystrobin in the leaf shredder *Gammarus fossarum* (Crustacea; Amphipoda) via two effect pathways. Ecotoxicol. Environ. Saf. 209, 1–9.

Appendix A3: Konschak, M., Zubrod, J.P., Baudy, P., Fink, P., Pietz, S., Duque Acosta, T.S., Bakanov, N., Schulz, R., Bundschuh, M., 2021. Mixture effects of a fungicide and an antibiotic: assessment and prediction using a decomposer-detritivore system. Aquat. Toxicol. 232, 1–8.

Appendix A4: Konschak, M., Zubrod, J.P., Duque Acosta, T.S., Bouchez, A., Kroll, A., Feckler, A., Röder, N., Baudy, P., Schulz, R., Bundschuh, M., 2021. Herbicide-induced shifts in the periphyton community composition indirectly affect feeding activity and physiology of the gastropod grazer *Physella acuta*. Environ. Sci. Technol. 55, 14699–14709.

Appendix A5: Curriculum vitae

Appendix A1

The importance of diet-related effects of the antibiotic ciprofloxacin on the leaf-shredding invertebrate *Gammarus fossarum* (Crustacea; Amphipoda)

Konschak, M., Zubrod, J.P., Baudy, P., Fink, P., Kenngott, K.G.J., Lüderwald, S., Englert, K., Jusi, C., Schulz, R., Bundschuh, M.

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Abstract

Antibiotics may constitute a risk for aquatic detritivorous macroinvertebrates (i.e., shredders) via waterborne and dietary antibiotic exposure. In addition, antibiotics can alter the food quality for shredders mediated by shifts in leaf-associated decomposer (i.e., aquatic fungi and bacteria) communities. However, little is known about the relative importance of the waterborne and dietary effect pathway. Therefore, we followed a tiered testing approach aimed at assessing the relative importance of these effect pathways. We employed the antibiotic ciprofloxacin (CIP) and the shredder *Gammarus fossarum* as model stressor and test species, respectively. In a first step, we assessed the short-term waterborne toxicity of CIP using survival and leaf consumption of *G. fossarum* as response variables. Alterations in the leaf-associated decomposer community, which may be reflected by their palatability, were assessed using food choice assays. Finally, we conducted a 2 × 2-factorial experiment over 24 days assessing the pathways individually and combined using energy processing (i.e., leaf consumption and feces production), growth and energy storage (i.e., neutral lipid fatty acids) as variables. Short term waterborne exposure indicated low toxicity with LC_{50} and EC_{50} values of 13.6 and 6.4 mg CIP/L, respectively. At the same time, shredders did not prefer any leaf material during the food choice assay. However, the fungal community was significantly affected in the highest CIP-treatments (0.5 and 2.5 mg/L) suggesting an altered food quality for shredders. This assumption is supported by the results of the long-term assay. At 0.5 mg CIP/L, gammarids' leaf consumption, growth and energy storage were increased when subjected via the dietary pathway, which was linked to changes in the leaf-associated microbial community. Our data highlight the importance of dietary effect pathways for effects on shredders, potentially impacting energy dynamics in detritus-based stream ecosystems.

Keywords

Aquatic fungi; Fatty acids; Fluoroquinolone; Food quality; Leaf litter breakdown

Introduction

Leaf litter is an important nutrient and energy source for detritus-based stream ecosystems (Nelson and Scott, 1962; Minshall, 1967; Fisher and Likens, 1973). Leaf-decomposing microorganisms (i.e., bacteria and fungi) as well as macroinvertebrate detritivores (i.e., shredders) play a pivotal role in its breakdown (Gessner et al., 1999; Graça, 2001). In this context, microbial decomposers (particularly aquatic hyphomycetes – a polyphyletic group of

asexual fungi; Baschien et al., 2006) provide two important functions: first, they make organic carbon accessible for local and downstream communities (Vannote et al., 1980) by degrading leaf litter (Hieber and Gessner, 2002; Baldy et al., 2007). Second, they increase the nutritional quality and palatability of leaf litter for shredders (i.e., microbial conditioning; Bärlocher and Kendrick, 1975b; Graça et al., 1993; Aßmann et al., 2011). Shredders, in turn, play a key role in transforming leaf litter into fine particulate organic matter, an important food source for collectors (Bundschuh and McKie, 2016), and are important prey for higher trophic levels (MacNeil et al., 1999).

The functional integrity of these aquatic decomposer-detritivore systems can, however, be influenced by chemical stressors (e.g., Rasmussen et al., 2012; Peters et al., 2013). As antibiotics, driven by their mode of action, affect bacteria (Brandt et al., 2015), they can influence leaf-associated microbial communities (Maul et al., 2006; Rico et al., 2014a). Effects on bacteria, may release aquatic fungi from competitive pressure for the same resources (Bundschuh et al., 2009) potentially altering leaves' nutritious quality and palatability for shredders (i.e., dietary effect pathway; Hahn and Schulz, 2007; Bundschuh et al., 2009). Furthermore, shredders may suffer from exposure to antibiotics via the water phase (i.e., waterborne effect pathway; Bartlett et al., 2013). However, relatively little is known about the relevance of the dietary and waterborne effect pathway for antibiotics (but see for fungicides e.g., Zubrod et al., 2015c). Recently, Bundschuh et al. (2017) reported effects of an antibiotic mixture on the feeding activity and physiology of a key shredder (i.e., *Gammarus fossarum* (Crustacea; Amphipoda)) in low-order streams of the northern hemisphere (Piscart et al., 2009) when subjected to both effect pathways. Although it was hypothesized that the dietary pathway was the main driver for these effects, a formal assessment of the effect pathways' relative importance is pending.

By targeting this knowledge gap, we focused on the model antibiotic ciprofloxacin (CIP; a DNA gyrase and topoisomerase IV inhibitor; Hooper and Wolfson, 1988) and its effects on *G. fossarum*. CIP was selected as it belongs to the group of fluoroquinolones, which have a broad range of applications in human and veterinary medicine (van Boeckel et al., 2014; European Medicines Agency, 2018). Furthermore, due to the high excretion of the non-metabolized parent compound through urine and feces (Mompelat et al., 2009) and its persistence during the wastewater treatment process (Batt et al., 2006), CIP is frequently detected at relatively high concentrations (up to the lower µg/L range) in surface waters compared to other antibiotics and is, according to the classification system of the European

Commission, toxic to very toxic for aquatic organisms (Danner et al., 2019). Using a tiered ecotoxicological testing approach, we first evaluated the waterborne toxicity of CIP recording gammarids' survival and feeding activity as response variable. Subsequently, a food choice assay was used to assess effects on the leaf-associated microbial community and the resulting impact on leaf palatability for *G. fossarum*. The shredder's food choice was employed as an indicator of resource quality (i.e., diet-related effects). Finally, we conducted a 24-day feeding assay to evaluate long-term waterborne and diet-related CIP effects on gammarids' energy processing (leaf consumption and feces production), growth and fatty acids (FAs) of triacylglycerols (TAGs, an important energy storage in invertebrates; Azeez et al., 2014) using a full-factorial (2 × 2) test design.

We expected that *G. fossarum* would be relatively insensitive towards CIP via waterborne exposure (cf. Park and Choi, 2008; Rico et al., 2014b) due to the high target specificity of antibiotics (Hooper and Wolfson, 1988). We, however, hypothesized that CIP would impair leaf-associated bacteria, which in turn release leaf-associated fungi from the competitive pressure by bacteria (Gulis and Suberkropp, 2003; Schneider et al., 2010). This would result in an increased fungal growth and hence increased palatability and resource quality for *G. fossarum* (Bundschuh et al., 2009), thus positively affecting the shredders' energy processing, growth and energy storage during the long-term feeding assay.

Materials and methods

Study designs

The assays were conducted in 2015 and 2016 with gammarids of the same population consisting of the cryptic lineage B (Feckler et al., 2012) and followed largely established protocols (Bundschuh et al., 2009; Zubrod et al., 2014; Zubrod et al., 2015b). For each assay, black alder (*Alnus glutinosa* (L.) GAERTN.) leaves were colonized with a near-natural microbial community serving as inoculum for the leaf material, which was used as food source for gammarids (Fig. 1). A 7-day feeding activity assay was conducted in September 2015 to assess the acute waterborne CIP toxicity to *G. fossarum*. The assay comprised six CIP concentrations (incl. a control) with 30 replicates each (cf. Zubrod et al., 2014; Fig. 1.A3). Nominal CIP concentrations (i.e., 0.5, 6.5, 12.5, 18.5, 24.5 mg/L) were derived from a range-finding test (a preliminary test to determine the concentration range before conducting the definite test). Although CIP concentrations are above field relevant levels, they were selected to determine toxicity parameters (e.g., the half-maximal effect concentration, i.e., EC_{50}),

which supported the selection of concentrations for the following assays as well as the interpretation of their data. In order to assess CIP-induced effect on leaves' food quality for shredders through changes in the leaf associated microbial communities, a 24-h food choice assay was performed in September 2015. The assay consisted of five CIP concentrations (i.e., 0, 20, 100, 500, 2500 µg/L) with 49 replicates each (cf. Bundschuh et al., 2009; Fig. 1. B3). The concentrations were based on reported concentrations of antibiotics showing significant effects on leaf-associated microorganisms (e.g., Maul et al., 2006; Bundschuh et al., 2009), while high concentrations were thought to enable the establishment of a dose-response relationship. Finally, an experiment using a 2 × 2-factorial test design was conducted in February 2016 to address the long-term waterborne and diet-related CIP effects on energy processing, growth and energy storage of *G. fossarum*. Therefore, shredders were subjected to (I) CIP-free test medium and non-exposed leaves, (II) waterborne CIP exposure and non-exposed leaves, (III) CIP-free test medium and CIP exposed leaves, (IV) waterborne CIP exposure and CIP exposed leaves (Fig. 1.C3). Each treatment consisted of 65 replicates with the CIP concentration (i.e., 0.5 mg/L) being selected on the basis of the other two experiments, namely to avoid gammarid mortality but still having impacts in leaf associated microorganisms.

Test substance

For the preparation of stock solutions, CIP (98 %, Acros Organics, Geel, Belgium) was dissolved in the respective test medium. Afterwards, the respective nominal concentrations were achieved (Table S1) by serial dilution in the respective test medium. Nominal CIP concentrations were verified by random sampling from three (feeding activity and food choice assay) or four (long-term feeding assay) replicates of the control, the lowest and highest test concentration at test start and after three days (only for long-term feeding assay). Samples were stored at −20 °C until analyses using an ultra-high-performance liquid chromatography system (Thermo Fisher Scientific, Bremen, Germany). Concentrations were determined via external standard calibration using matrix-aligned standards (cf. Zubrod et al., 2015c). As all measured CIP concentrations, except for the lowest treatment of the feeding activity assay, deviated by less than 20 % from nominal concentrations (Table S1), the latter are reported throughout this manuscript.

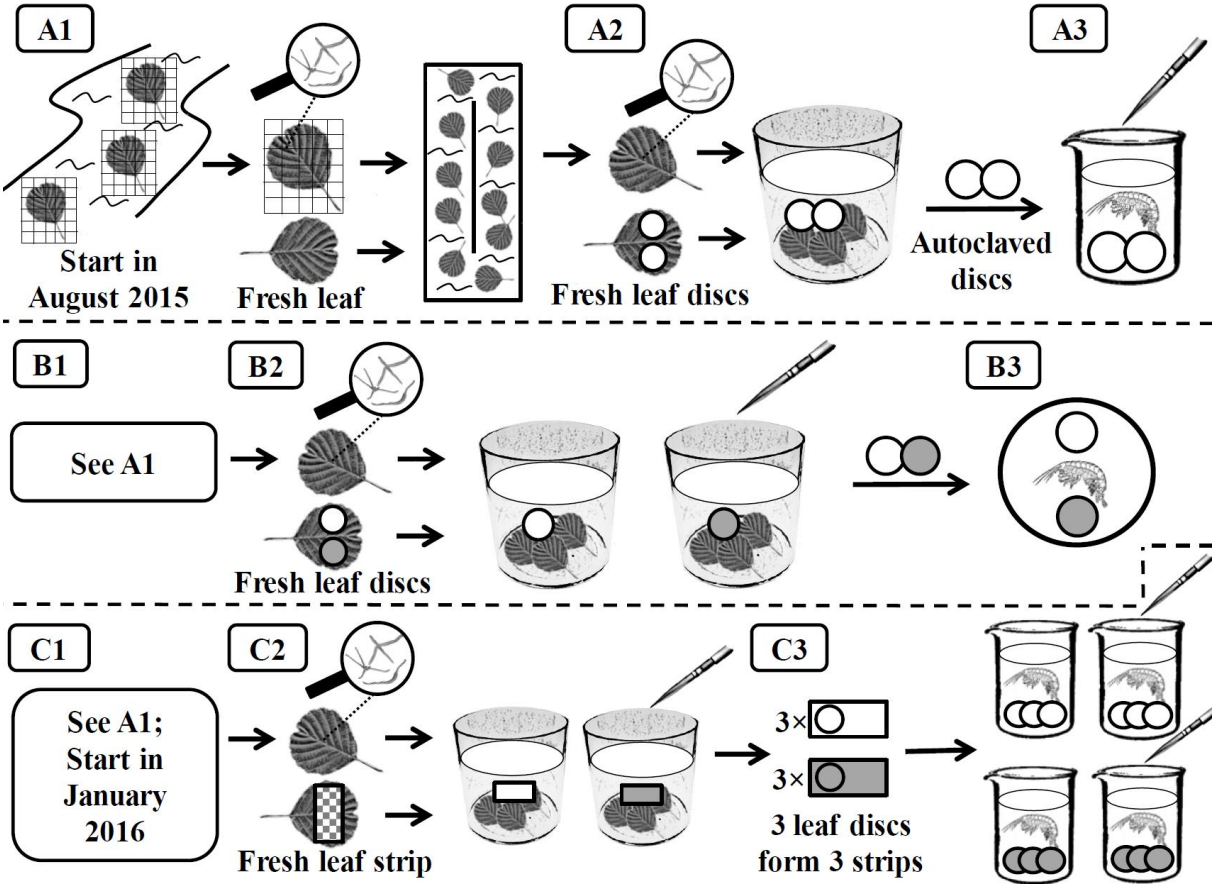

Fig. 1 Schematic overview of the three test designs (**A**, **B** and **C**). Before starting each assay, fresh leaves were deployed for 14 days in a stream (to establish a leaf-associated microbial community) followed by a 14-day conditioning process with microbially colonized and fresh leaves in a stainless-steel container under laboratory conditions (**A1**, **B1** and **C1**). **A2**, **B2** and **C2** describe the conditioning process of leaf discs or strips (cut from fresh leaves) in the absence and presence (denoted by the pipette) of CIP. **A3**, **B3** and **C3** display the experimental setup of each assay: **A3** illustrates the experimental setup of the 7-day feeding activity assay where *G. fossarum* was subjected to waterborne CIP exposure (denoted by the pipette). **B3** displays the 24-hour food choice assay where *G. fossarum* was offered leaf discs, which were microbially conditioned in the absence or presence of CIP (denoted by white and grey discs, respectively). **C3** shows the 2 × 2-factorial test design of the 24-day long-term feeding assay with the first factor being the absence or presence of waterborne CIP exposure (denoted by the absence or presence of the pipette). The second factor was leaves serving as food for *G. fossarum*, which were microbially colonized in the absence or presence of CIP (denoted by white and grey discs, respectively).

Sources of leaves, microorganisms, and gammarids

As described in Bundschuh et al. (2011), black alder leaves were handpicked from a group of trees near Landau, Germany (49°20´N; 8°09´E) in October 2015 and stored at −20 °C. Before the start of each assay, defrosted black alder leaves were colonized with a near-natural lotic microbial community by deploying leaf material in mesh bags (mesh size ~1mm) for 14 days in the stream Rodenbach, Germany (49°33´N; 8°02´E) upstream of agricultural land use and effluent discharges. Back in the laboratory, the microbially colonized leaves were combined with unconditioned black alder leaves in a stainless-steel container filled with 30 L of conditioning medium (Dang et al., 2005) and left at 16 ± 1 °C, under permanent aeration and in total darkness for further 14 days before being used as microbial inoculum.

As described in Zubrod et al. (2010), seven days before the start of each assay, individuals of *G. fossarum* were collected in the stream Hainbach, Germany (49°14´N; 8°03´E) upstream of agricultural land use and effluent discharges. Back in the laboratory, gammarids were divided into size classes via a passive separation technique (Franke, 1977). To reduce within-treatment variation, only males (sex was identified by position in pre-copula pairs) with a cephalothorax length of 1.2 – 1.6mm and uninfested by acanthocephalan parasites (Pascoe et al., 1995; Fielding et al., 2003) were used for the assays. Test organisms were gradually acclimatized at 16 ± 1 °C in total darkness to the amphipod culture medium SAM-5S (Borgmann, 1996), which was used as test medium. Gammarids were fed *ad libitum* with microbially conditioned black alder leaves. To stimulate their appetite for the food choice assays, gammarids were not fed 96 h before test start.

Feeding activity assay

Leaf discs of 20 mm diameter were cut from unconditioned black alder leaves using a cork borer, inserted into mesh bags and conditioned at 16 ± 1 °C in total darkness by using 15-L aquaria containing 12 L of conditioning medium and 50 g (wet weight) of microbial inoculum as described by Zubrod et al. (2014). After 10 days, leaf discs were dried at 60 °C for 24 h, autoclaved (to avoid unintended indirect, diet-related effects on *G. fossarum*; model DE-65®, Systec, Linden, Germany), dried again for 24 h and weighed in sets of two discs to the nearest 0.01 mg. Before the start of the assay, discs were re-soaked with autoclaved SAM-5S for 48 h to reduce buoyancy.

The 7-day assay was conducted in total darkness and at 16 ± 1 °C. Each replicate comprised a permanently aerated 250-mL glass beaker containing 200 mL of SAM-5S, a set

of two autoclaved leaf discs, and one gammarid. Five additional beakers were set up without animals to allow the quantification of microorganism-induced and handling-related leaf mass loss. After 7 days, dead animals were recorded. Animals and leaf disc remains from replicates with surviving gammarids were dried and weighed as described above.

Food choice assay

As described by Bundschuh et al. (2009), sets of four leaf discs of 16 mm diameter were cut from single unconditioned black alder leaves and subsequently dried for 24 h at 60 °C, weighed individually to the nearest 0.01 mg, and re-soaked with autoclaved SAM-5S for 48 h. Afterwards, two discs of each set were placed into pockets of an individually labeled mesh bag and were microbially colonized (at 16 ± 1 °C and in total darkness) for 12 days in 5-L aquaria containing 4 L of CIP-free aerated conditioning medium (i.e., control) and 10 g (wet weight) of microbial inoculum. The remaining two discs of the same set were conditioned under the same conditions, but in the presence of one of four CIP concentrations (n = 7). To ensure a continuous exposure over the 12-day conditioning phase, the conditioning medium as well as the respective CIP concentration were renewed every third day. At the end of the conditioning process, leaf discs were rinsed for 30 min in CIP-free SAM-5S and immediately introduced into the food choice assays or preserved for microbial analyses.

Each food choice assay (cf. Bundschuh et al., 2009) comprised 49 crystallization dishes filled with 100 mL of SAM-5S. In each dish, one gammarid was offered one leaf disc microbially colonized under control conditions and one disc of the same leaf disc set, which was colonized in presence of one of the four CIP concentrations. The remaining two leaf discs of the same set, which were inaccessible for the gammarid in the crystallization dish (see Bundschuh et al., 2009 for a schematic representation of a feeding arena), served for the quantification of microbial leaf litter decomposition over the whole experimental duration (i.e., conditioning phase and food choice assay). Assays lasted for 24 h and were performed at 16 ± 1 °C in total darkness. At the end of each experiment, surviving animals and leaf disc remains were dried for 24 h at 60 °C and subsequently weighed to the nearest 0.01 mg. Replicates with gammarids that had died or escaped from the test arena were excluded from further analyses.

Long-term feeding assay

As described by Zubrod et al. (2015b), leaf strips (~10 × 5 cm) were cut from unconditioned black alder leaves. Enclosed in mesh bags, the strips were microbially colonized for 12 days

under the same conditions as described for the feeding activity assay in absence (i.e., control) or presence of 0.5 mg CIP/L (n = 3). The conditioning medium was renewed every third day to ensure a continuous antibiotic exposure. After 12 days, one set of two leaf discs of 20 mm diameter was cut from each of the 130 strips per aquarium excluding the leaves' midrib and three sets (one per aquarium) were immediately introduced into each test vessel of the assay. To ensure *ad libitum* feeding on fresh leaf material over the 24 days, four independent 12-day leaf conditionings were started at intervals of 6 days. During each food renewal, additional leaf discs of 16 mm diameter were cut and preserved for FA and microbial analyses.

Using a 2 × 2-factorial test design (cf. Zubrod et al., 2015b), gammarids were either subjected to a control treatment (Control), to waterborne CIP exposure (Water), a treatment, where the animals received leaves that were microbially conditioned in the presence of CIP (Diet), or a combination of the two effect pathways (Combined; see Fig. 1.C3). Replicates of each treatment (n = 65) comprised a 250-mL glass beaker filled with 200 mL of SAM-5S that was continuously aerated. Each beaker was equipped with one gammarid kept in a cylindrical mesh cage made from stainless steel with a mesh size of 0.5 mm (to guarantee a careful transfer of the animals into new test vessels during medium exchanges). Animals were allowed to feed on three leaf discs from different sets. The three corresponding leaf discs from the same sets were deployed in the beakers within rectangular stainless steel mesh cages that prevented feeding by the gammarids and hence allowed to control for microbial and handling-related leaf mass loss. The two cages were separated by a watch glass to prevent the interaction of animal's feces with the leaf discs in the rectangular cage (see Zubrod et al., 2015b for a schematic representation of an assay replicate). Every third day, SAM-5S as well as the CIP concentration in the respective treatment was renewed, to guarantee a chronic exposure, and dead animals were recorded and discarded. In addition, to quantify the amount of gammarids' feces, the 3-day old SAM-5S containing the animals' feces was filtered through pre-weighed glass fiber filters (GF/6, Whatman, Dassel, Germany), which were used twice within each 6-day interval and stored at 60 °C. Every sixth day, leaf disc remains of both cages were replaced by freshly conditioned leaf discs. Leaf disc remains and filters were dried and weighed as described above. To correct for changes in filter weight by handling and microbial and physico-chemical leaf mass loss (as both can cause the formation of fine particulate organic matter), three additional replicates without test organism were set up per treatment. At the end of the experiment, gammarids were shock-frozen in liquid nitrogen and stored at −80 °C before being freeze-dried and weighed to the nearest 0.01 mg. Replicates

containing dead animals (8, 5, 14 and 8 % in the Control, the Water, the Diet and the Combined treatment, respectively) were excluded from further statistical analyses.

Microbial analyses

To shed light on mechanisms underlying CIP-induced alterations of the microorganism-mediated food quality for *Gammarus* (i.e., indirect effects), microbial parameters (i.e., ergosterol content, bacterial densities and hyphomycete community structure) were analyzed. During the food choice and long-term feeding assay, 15 leaf discs of 16 mm diameter and five leaf strips, respectively, of each aquarium (i.e., $N = 35 = 7$ replicates × 5 treatments and $N = 24 = 3$ replicates × 2 treatments × 4 independent leaf conditionings) were stored at −20 °C for analysis of ergosterol. Moreover, during both assays, three leaf discs (diameter = 16 mm) per aquarium were preserved in a 2 % formaldehyde/ 0.1 % sodium pyrophosphate solution and stored at 4 °C for quantification of bacterial densities. Furthermore, for the determination of the hyphomycete community structure, five leaf discs (diameter = 16 mm) were shaken (120 rpm) in deionized water for 96 h (at 16 ± 1 °C and in total darkness) to stimulate sporulation of fungi and preserved in a 2 % formaldehyde/0.5 % polysorbate 80 (Tween® 80, Carl Roth, Karlsruhe, Germany) solution at 4 °C for later analysis.

Ergosterol content was analyzed according to Gessner and Schmitt (1996). This sterol occurs in cell membranes of Eumycota and is considered as a proxy for leaf-associated fungal biomass (e.g., Gessner, 2005). Ergosterol was extracted via solid-phase extraction (Sep-Pak® Vac RC tC18 500 mg sorbent, Waters, Milford, US-MA) and measured by high-performance liquid chromatography (1200 Series, Agilent Technologies, Santa Clara, US-CA) using a LiChrospher® 100 RP-18 column (250mm × 4.6 mm, particle size 5 µm, Merck Millipore, Billerica, US-MA). Ergosterol concentration was quantified via external calibration curve and normalized to leaf dry mass.

Bacterial densities were quantified according to Buesing (2005). Briefly, bacterial cells were detached from the discs using ultrasonicationand subsequently stained via SYBR® Green II (Molecular Probes, Eugene, US-OR). The number of cells was determined by using a fluorescence microscope and the software AxioVision (Axio Scope.A1, AxioCam MRm and AxioVision Rel. 4.8, Carl Zeiss MicroImaging, Jena, Germany). The mean number of 20 digital photographs was extrapolated to the total sample volume and normalized to leaf dry mass by drying and weighing (as described above) of three additional leaf discs per sample

from the same aquarium and the same leaf strips for the food choice and long-term feeding assay, respectively.

Following Pascoal and Cássio (2004), the hyphomycete community structure was determined by identifying species via spore morphology. Therefore, fungal spores were fixed on a cellulose filter (S-Pak Filters 0.45 µm, 47 mm white gridded, Merck Millipore, Billerica, US-MA) and stained with a cotton blue solution. Subsequently, spores were identified using a microscope as well as various identification keys (e.g., Ingold, 1975). Afterwards spores were extrapolated to the total sample volume and normalized to leaf dry mass.

Fatty acid analyses

We quantified TAG FAs (i.e., neutral lipid fatty acids, NLFAs) in gammarids, as they constitute the major energy storage in invertebrates (Azeez et al., 2014) and their composition can be affected relatively quickly (compared to phospholipid fatty acids) by changes in the diet (Iverson, 2012). Additionally, we analyzed NLFAs of conditioned leaf materials to determine fungi-mediated alterations in the food quality for shredders (fungi pose a crucial quality parameter to the shredder's diet; e.g., Graça et al., 1993), since TAGs constitute the major lipid class in fungi (Harwood and Russell, 1984). Although many prokaryotes are incapable to store energy in this form (Alvarez and Steinbüchel, 2002), background NLFA concentrations originating from bacteria cannot be completely excluded (Bååth, 2003).

For NLFA quantification, ten gammarids of each treatment and portions of five different leaf strips (in total ~40 mg leaf dry weight) per aquarium ($N = 40$ and $N = 24$, respectively) were freeze-dried and weighed as described above. The extraction and purification of lipids were performed according to Bligh and Dyer (1959) with slight alterations: gammarids were homogenized in a chloroform/methanol/ water mixture (1:2:0.8) using an Ultra-Turrax blender (at 6500 rounds/ min for few seconds; T25 basic, IKA® Werke GmbH & Co. KG, Staufen, Germany) and leaf material was crushed manually before the chloroform/methanol/water mixture was added. Afterwards, a TAG with three deuterated 18:0 FAs (Tristearin-D105, Larodan, Solna, Sweden) as internal standard as well as chloroform and water (to obtain the mixture ratio of 2:2:1.8; cf. Bligh and Dyer, 1959) were added to each sample and the samples were stored overnight at 4 °C. TAGs were separated from glycolipids and phospholipids by elution with 4 mL chloroform through conditioned (with 4 mL chloroform) solid phase extraction columns (Chromabond® easy polypropylene columns, Macherey-Nagel, Düren, Germany). Afterwards, the solvent was evaporated under nitrogen in

a dry heat incubator (VLM Metallblockthermostate, VLM GmbH, Bielefeld, Germany) at 40 °C and TAGs were subsequently solved in 100 µL of chloroform. According to Butte (1983), NLFAs were transesterified by trimethylsulfonium hydroxide (Sigma-Aldrich, St. Louis, US-MO) and the resulting fatty acid methyl esters (FAMEs) were analyzed using a gas chromatograph (CP-3800, Varian, Palo Alto, US-CA) equipped with a flame ionization detector and a DB-225 GC column (30 m, ID 0.25 mm, film thickness 0.25 µm, J &W Scientific, Folsom, US-CA; cf. Fink, 2013). Nitrogen was used as carrier gas. FAMEs in each sample were determined using the retention times of FAME standards (Sigma-Aldrich, St. Louis, US-MO) and FAs were quantitatively analyzed via external standard calibration (i.e., µg FA/mL). NLFA concentrations were adjusted for FA traces originating from solvents using extraction blanks. Furthermore, concentrations of the FAs were corrected using the respective internal standard's recovery rate. The corrected FA concentrations were extrapolated to the total sample volume and normalized to sample weight (i.e., mg FA/g dry sample mass).

Calculations and statistics

The leaf material consumed by G. fossarum during the feeding activity and food choice assays was expressed as mg consumed leaf material/mg individual/day and calculated as described by Naylor et al. (1989) and Bundschuh et al. (2009), respectively. Microbial decomposition of the inaccessible leaf discs for *G. fossarum* during the food choice assay was expressed as mg leaf mass loss/day and calculated according to Zubrod et al. (2015a). For the 24-day long-term feeding assay, leaf consumption in mg/day was calculated as per Zubrod et al. (2011). Gammarid growth in µg/day was defined as dry mass gain and derived by subtracting the mean dry mass of 48 gammarids shock-frozen at the test start from the final dry mass of each individual divided by 24 days.

Effect concentrations resulting in 20 and 50 % mortality and inhibition of leaf consumption (LC_{20}/EC_{20} and LC_{50}/EC_{50} values) were determined by fitting various concentration-response models to the feeding activity assay's data. The models with the best fit were selected based on Akaike's information criterion (Table S2). Prior to null hypothesis significance testing (NHST), extreme values were detected by visual inspection of boxplots (with a 1.5 × interquartile range) and excluded from further analyses only when they differed considerably from the main trend of data (Field et al., 2012). Normality and homoscedasticity were tested using the Shapiro–Wilk test and Levene's test, respectively, as well as visual inspection. When both presumptions of parametric testing were met, unpaired data from one-

way designs with two factor levels and at least three factor levels were analyzed using Student's *t*-test and analysis of variance (ANOVA) followed by Dunnett's test, respectively. Data from the 2 × 2-factorial design of the long-term feeding assay were analyzed via two-way ANOVA. Paired data were evaluated using paired *t*-tests. When one of the assumptions for parametric testing was violated, Wilcoxon rank-sum and Wilcoxon signed-rank tests were used for unpaired and paired data from one-way designs, respectively, followed by a Bonferroni correction for multiple comparisons if more than two factor levels were tested (Zar, 2010). For non-parametric data from the long-term feeding assay, data were rank-transformed before performing a two-way ANOVA (Conover and Iman, 1981) or the Brunner-Dette-Munk test (sensu Aho, 2019) was applied, if the assumption of homoscedasticity was still violated after ranking (Brunner et al., 1997). Multivariate data were square-root transformed, to decrease the discriminatory power of dominant sporulating fungal species and NLFAs (Happel et al., 2017), and tested via permutational multivariate analysis of variance (PERMANOVA). For the visualization of (dis-)similarities of the hyphomycete communities as well as NLFA composition, data were displayed via non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarity. Fungal sporulation data were zero-adjusted by adding a dummy species with an abundance of one to each replicate (Clarke et al., 2006) to determine Bray-Curtis dissimilarities.

Detailed information on NHST (i.e., *p*-values, *F*-statistics, sum and mean of squares as well as group medians with 95 % confidence intervals) of the assays are provided in Table 1 and S3–S9. Modeling, statistics and figures were conducted with R Version 3.5.1 for Windows (R Core Team, 2014) as well as the add-on packages, "asbio", drc", "multcomp", "plotrix" and "vegan". Note that the term "significant" refers to statistical significance throughout the study.

Results and discussion

Short-term waterborne effects

During the 7-day feeding activity assay, exposure to CIP resulted in a concentration-dependent increase and reduction in mortality and leaf consumption of *G. fossarum*, respectively (Fig. 2, Table S3). The EC_{50} values for survival and leaf consumption were 13.6 and 6.4 mg CIP/L and the respective EC_{20} values were 9.5 and 0.5 mg CIP/L. Since the acute CIP toxicity for *G. fossarum* is in the mg/L range, which is comparable with reported toxicity data for *Daphnia* spp. (Martins et al., 2012; Dalla Bona et al., 2014), crustaceans, in general,

seem to be relatively tolerant to waterborne CIP exposure. However, prokaryotes and unicellular eukaryotes are often more sensitive towards antibiotics than invertebrates (Danner et al., 2019). Consequently, we expected effects on leaf-associated microorganisms at lower CIP concentrations during microbial conditioning.

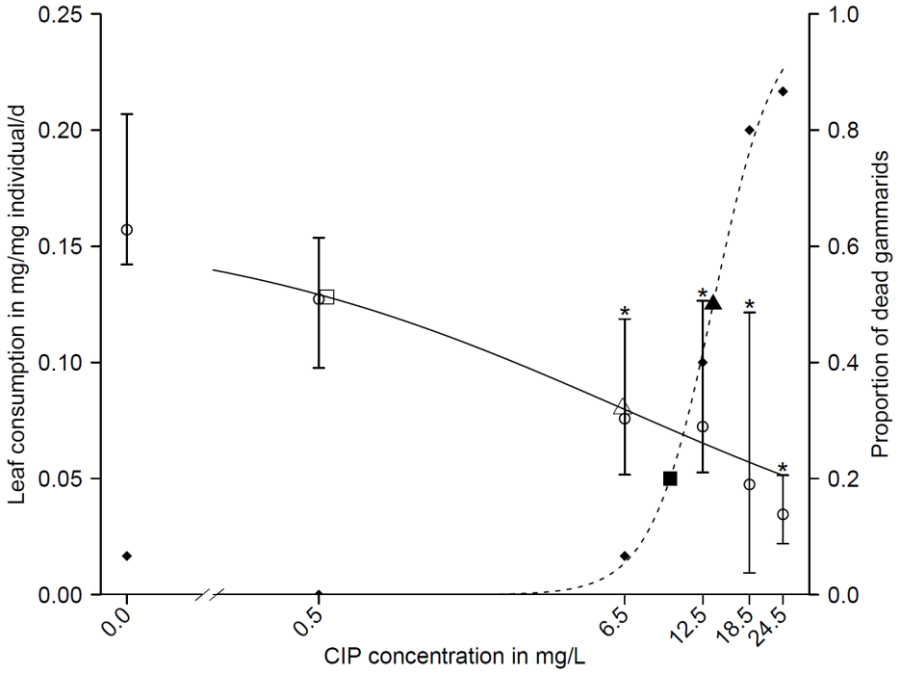

Fig. 2 Median leaf consumption (open circles with 95 % CIs) of *G. fossarum* and proportion of dead gammarids (solid diamonds) when subjected to increasing CIP concentrations. Moreover, the models with the best fit (solid line for leaf consumption and dashed line for mortality) as well as the EC_{20}/LC_{20} (transparent and solid squares, respectively) and EC_{50}/LC_{50} values (transparent and solid triangles, respectively) are displayed. Asterisks indicate a statistically significant difference to the control.

Food choice – a proxy for dietary effects

In line with our expectations, leaf-associated microorganisms were affected at CIP concentrations, which were five-fold below those negatively affecting the leaf-shredding invertebrate (Table S4). Contrary to our hypothesized release of competitive pressure for leaf associated fungi, however, fungal biomass (measured as ergosterol) was significantly reduced by ~55 and ~60 % at 500 and 2500 µg CIP/L, respectively, while bacterial density was not significantly affected (Table S4). The latter may be explained by the unexpected negative impact on aquatic fungi, which probably reduced the competitive pressure for bacteria. This relief of competition coupled with a relatively fast adaptation of the bacterial community to chemical stress (e.g., replacement of sensitive species and evolutionary acquisition of CIP

resistance; Brandt et al., 2015), may explain the results at the highest CIP concentrations. Moreover, the community composition of hyphomycetes was significantly shifted when exposed to 100 µg CIP/L (Fig. 3, Table S4). Similar to fungal biomass, these alterations in the community structure constitute an indicator for chemical stress-induced shifts in the palatability of leaf litter as well as its quality for shredders (Bundschuh et al., 2011). This can be assumed as fungal species vary in their palatability and nutritional value for amphipod shredders (Bärlocher and Kendrick, 1973, Arsuffi and Suberkropp, 1989, Aßmann et al., 2011). The significant community shift was mainly driven by direct effects on *Fusarium* sp., the most prevalent hyphomycete species associated with leaves during the food choice assay (Table S5). Indeed, fluoroquinolones show antifungal activities on the same genus (causing fungal keratitis) by inhibiting type II topoisomerase DNA gyrase and topoisomerase IV (e.g., Day et al., 2009).

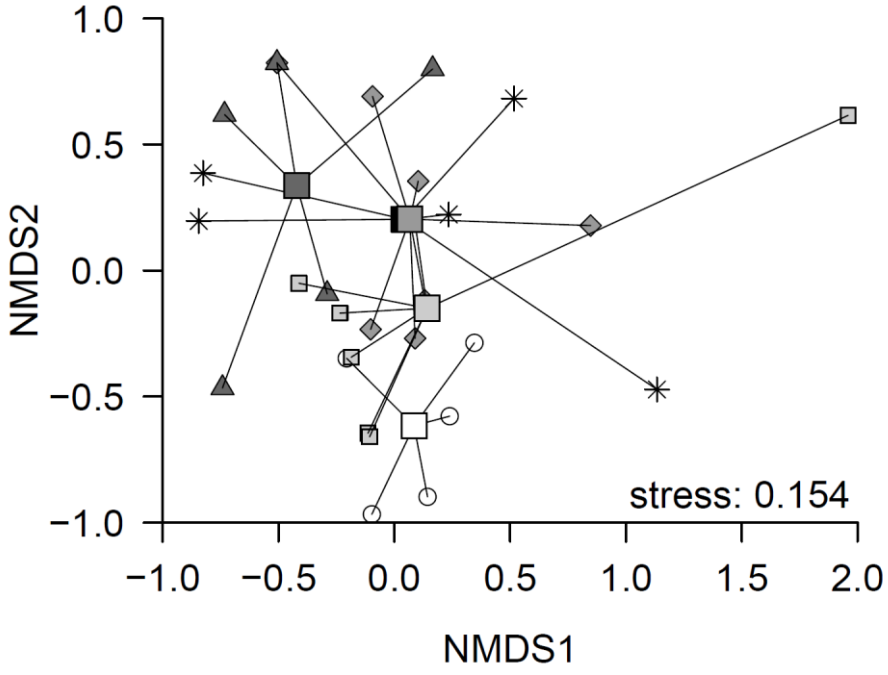

Fig. 3 Non-metric multidimensional scaling (NMDS) plot for hyphomycete communities associated with leaf material conditioned under control conditions (white circles) and in the presence of 20 (light grey squares), 100 (grey diamonds), 500 (dark grey triangles) and 2,500 (black asterisks) µg CIP/L during the food choice assay. A stress value is provided as a measure of "goodness-of-fit" for NMDS with reasonable fits indicated when below 0.2 (Clarke, 1993).

In contrast to our hypotheses and despite these CIP-induced shifts in the microbial community, *G. fossarum* did not show significant preferences during the food choice assay (Fig. 4, Table S4) indicating that the reduction in *Fusarium* sp. is not mirrored in the leaves'

palatability. However, consumption of this fungus was shown to increase the nutritional value of leaves and affect shredders' growth positively (Bärlocher and Kendrick, 1973, Bärlocher and Kendrick, 1975a). Accordingly, we expected indirect negative implications on the gammarids' growth and energy storage via the dietary pathway over the long run triggered by a lower nutritious quality.

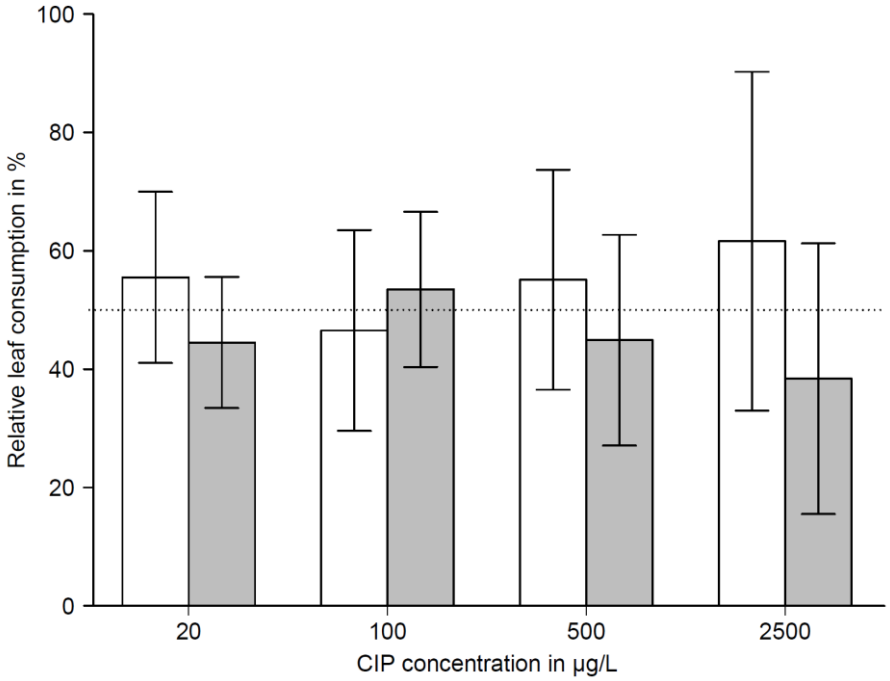

Fig. 4 Mean relative leaf consumption (with 95 % CIs) of *G. fossarum* on leaves microbially colonized under control conditions (white bars) or in the presence of increasing CIP concentrations (grey bars). The dotted line indicates the no-effect level (i.e., 50 % consumption on both leaf types).

Long-term waterborne and diet-related CIP effects

In accordance with our initial hypothesis but contrary to the results of the food choice assay (see 3.2), 0.5 mg CIP/L significantly elevated the leaf consumption (~20 %) of *G. fossarum* via the dietary pathway, while feces production was not affected (Fig. 5, Table 1). The increased leaf consumption might be explained by a CIP-induced higher food quality, as, in this assay, ergosterol content (i.e., a proxy for fungal biomass) was significantly increased when leaves were conditioned in presence of CIP (Table S6). The higher fungal biomass might have stimulated the leaf consumption of gammarids (Foucreau et al., 2013), ultimately resulting in a tendency to higher growth (~50 %) and energy storage (i.e., NLFA content, ~10 %; Figure 3 and 4, Table 1) of gammarids. As originally hypothesized, the observed responses in the leaf-associated microbial community and ultimately *G. fossarum* may be

driven by giving the leaf-associated fungi a competitive advantage through the impact of CIP on bacteria. This hypothesis could be (alongside the increased ergosterol content) supported by a non-significant reduction of the leaf associated bacterial density (~25 %; Table S6). However, the increased fungal biomass was not reflected by typical fungal FA markers (18:1ω9 and 18:2ω6; Bååth, 2003; Table S9). This might be explained by fungi investing energy preferably in growth rather than in energy storage under the provided conditions (i.e., a surplus of carbon, nitrogen, and phosphorus from both leaves and the conditioning medium; Bååth, 2003).

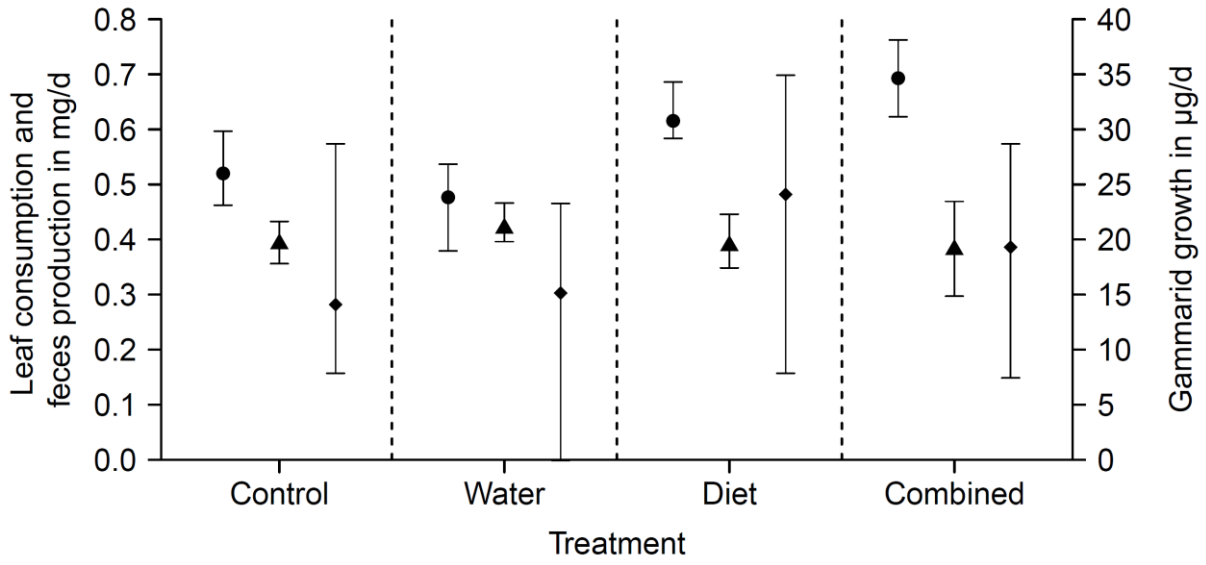

Fig. 5 Median (with 95 % CIs) leaf consumption (points), feces production (triangles) and growth (diamonds) of *G. fossarum* subjected to different effect pathways during the long-term feeding assay with CIP. Statistical analyses are displayed in Table 1.

The contrasting effects on the leaf-associated microbial communities in this experiment compared to the food choice assay (see 3.2) are likely related to the utilization of microbial inocula from different seasons leading to a different species composition (Nikolcheva and Bärlocher, 2005). While there were no adverse effects on any of the hyphomycete species in the long-term feeding assay (Table S7), sporulation of *Fusarium* sp. was substantially affected during the food choice assay and ergosterol content was significantly reduced at 0.5 mg CIP/L. These differing effects observed with the field collected leaf associated microbial community point towards their high plasticity motivating further studies targeting the underlying mechanisms.

Table 1 ANOVA-tables for all gammarid-related endpoints during the long-term feeding assay. All *p*-values <0.05 are printed in bold.

Endpoint	Factor	df1	SS/df2	MS/R2	*F*-value	*p*-value	ANOVA type
Leaf consumption	Water	1	0.016	0.0163	0.295	0.588	Two-way ANOVA
	Diet	1	1.478	1.4783	26.764	**<0.001**	
	Water × Diet	1	0.260	0.2598	4.703	**0.031**	
	Residuals	233	12.869	0.0552			
Feces production	Water	1	222.6602	-	0.187	0.666	Brunner-Dette-Munk test
	Diet	1	222.6602	-	1.015	0.315	
	Water × Diet	1	222.6602	-	1.456	0.229	
	Residuals	-	-	-			
Growth	Water	1	0.00000	0.000001	0.000	0.983	Two-way ANOVA
	Diet	1	0.00361	0.003611	2.959	0.087	
	Water × Diet	1	0.00046	0.000457	0.374	0.541	
	Residuals	232	0.28311	0.001220			
Total FA content	Water	1	539	539.2	1.747	0.195	Two-way ANOVA
	Diet	1	1190	1190.0	3.856	0.057	
	Water × Diet	1	711	711.3	2.305	0.138	
	Residuals	36	11110	308.6			
SAFA content	Water	1	128.0	128.01	3.492	0.070	Two-way ANOVA
	Diet	1	174.1	174.12	4.750	**0.036**	
	Water × Diet	1	49.9	49.87	1.360	0.251	
	Residuals	36	1319.7	36.66			
MUFA content	Water	1	61.4	61.44	0.965	0.333	Two-way ANOVA
	Diet	1	197.4	197.37	3.099	0.087	
	Water × Diet	1	190.6	190.55	2.992	0.092	
	Residuals	36	2293.0	63.69			
PUFA content	Water	1	16.6	16.55	0.865	0.358	Two-way ANOVA
	Diet	1	52.6	52.59	2.749	0.106	
	Water × Diet	1	33.7	33.69	1.761	0.193	
	Residuals	36	688.7	19.13			
FA composition of gammarids	Water	1	0.012946	0.05297	2.312	0.111	PERMANOVA
	Diet	1	0.020477	0.08379	3.656	**0.047**	
	Water × Diet	1	0.009353	0.03827	1.670	0.193	
	Residuals	36	0.244392	0.82497			

Moreover, CIP tends to adsorb to organic carbon (log K_{OC} of ~4 – 5 L/kg at neutral pH, Cardoza et al., 2005; Belden et al., 2007), which may ultimately increase internal CIP concentrations in *G. fossarum* via the dietary uptake. Through this pathway, a shift in the microbiome of the shredder's gut may have been induced (see for antibiotic effects on the invertebrates' gut microbiome Gorokhova et al., 2015 and Zhu et al., 2018). The gut microbiome is involved in energy harvest by transforming the components of the diet into

easily digestible substances (Cani et al., 2008) and in the regulation of appetite hormones (Mu et al., 2016). A potential stimulation of the appetite (Perić-Mataruga et al., 2009) and thus leaf consumption may have enhanced growth and energy storage. Moreover, the positive effects on *G. fossarum* could also be explained by antibiotics actively dampening immune responses, thereby reducing energy costs of the animal's immune system (see for vertebrates e.g., Niewold, 2007 and Brown et al., 2017). Thus, the energy surplus (due to the reduction of immune responses) could have resulted in increased energy allocation to gammarids' (feeding) activity and growth. However, the immunobiology (Loker et al., 2004) and the gut microbiome-host interactions (Lee and Hase, 2014) in invertebrates are not understood well enough yet to draw final conclusions on CIP as growth promoter in *G. fossarum*.

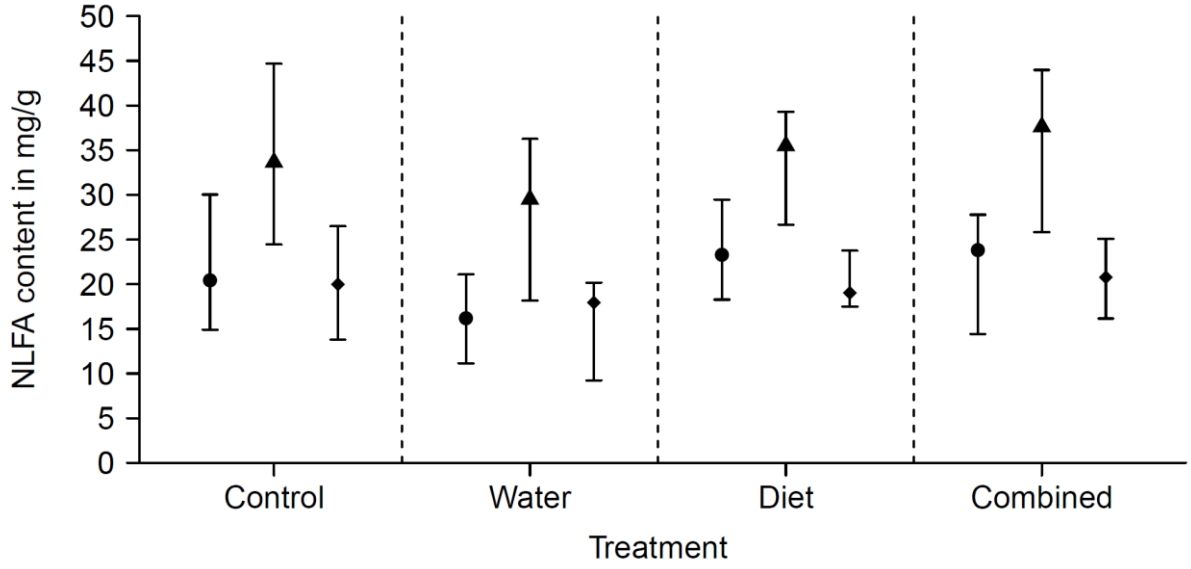

Fig. 6 Median (with 95 % CIs) saturated (SAFA; points), monosaturated (MUFA; triangles) and polysaturated (PUFA; diamonds) fatty acid content of *G. fossarum* subjected to different effect pathways during the long-term feeding assay with CIP. Statistical analyses are displayed in Table 1.

Contrary to the diet-related effect pathway, waterborne CIP exposure did not affect the gammarids' leaf consumption, feces production or growth (Fig. 5, Table 1). The content of saturated fatty acids (SAFAs) – mainly those with a shorter carbon chain length (i.e., 12:0 – 17:0; Table S8) – were reduced non-significantly (mono- (MUFAs) and polyunsaturated fatty acids (PUFAs) were not affected; Fig. 6, Table 1).These shorter FAs tend to be mobilized relatively quickly in situations of energy shortage (e.g., during starvation; Werbrouck et al., 2016; Price and Valencak, 2012). As CIP can induce the production of reactive oxygen species (ROS; Wang et al., 2018), defense mechanisms could increase the organism's energy

demand (Sokolova et al., 2012), which would explain the observed lower SAFA levels. Proteomic analyses (e.g., via mass spectrometry-based proteomics; Sokolowska et al., 2011) may help to link CIP-exposure with the induction of respective stress proteins unraveling the underlying physiological mechanisms.

When the dietary pathway acted jointly with waterborne CIP exposure, a significant synergistic interaction was observed for gammarids' leaf consumption (no interactions were observed for the remaining endpoints Fig. 5 and 6, Table. 1). This synergism was derived from the fact that the change in leaf consumption in the Combined treatment cannot be explained by summing up the effects induced by the individual pathways alone. It is likely that CIP originating from the water phase additionally adsorbed to the food already conditioned in the presence of CIP. Consequently, the gammarids' exposure through the gut was potentially further increased, thus exacerbating the effects on the gut microbiome and shredder's immune system. This hypothesized effect cascade is supported by the measured water concentrations, showing a 30 % reduction of CIP between water exchanges (measured in Water treatment; Table S1). Therefore, adsorbed CIP may have resulted in an intensification of the diet-related effects in *G. fossarum*. Moreover, positive diet-related effects seem to even cancel out the negative waterborne effects, since no (significant) reduction of the energy storage was observed (Fig. 6, Table. 1). In summary, our data suggest that CIP can affect growth and energy storage, respectively, of *G. fossarum* via waterborne and dietary exposure as well as via CIP-induced alterations of the microorganism-mediated food quality and the shredder's gut microbiome. Furthermore, diet-related effects outweigh waterborne effects, when both pathways act jointly.

Environmental relevance

The present study shows that CIP concentrations altering leaf-associated microbial communities and thus potentially affecting *G. fossarum* via the dietary pathway are in the high µg/L range, while CIP concentrations at least one order of magnitude higher are needed to induce direct effects through waterborne exposure. As experimental concentrations are several orders of magnitude beyond concentrations usually detected in European surface waters (Danner et al., 2019), the present study suggests a low risk for decomposer-detritivore systems. Nonetheless, Bundschuh et al. (2017) showed that comparable effects can be induced by an antibiotics mixture at 2 µg/L being a typical exposure scenario nowadays (e.g., Riva et al., 2019). Moreover, antibiotic concentrations in surface waters are projected to increase due to the growing population, increasing economic growth, and the expansion of the medical

sector (van Boeckel et al., 2014; Klein et al., 2018). At the same time, inadequate wastewater management, particularly of pharmaceutical industries, lead in extreme cases to concentrations in the mg/L range (e.g., 2.5 mg CIP/L; Fick et al., 2009). All in all, these insights warrant the consideration of antibiotics as a potential stressor interacting with decomposer-detritivore systems and thus to understand the underlying mechanisms leading to effects.

Conclusion

The present study shows that effects of the model antibiotic CIP via the dietary pathway seem to be more relevant for *G. fossarum* than waterborne antibiotic effects. The dietary pathway in isolation and both effect pathways in combination resulted in a higher turnover rate of leaf litter by the shredders, which may influence carbon and energy dynamics in detritus-based ecosystems due to their bottom-up regulation (Wallace et al., 1997, Johnson and Wallace, 2005). As leaf litter input to streams occurs mainly in autumn and serves as between-year food storage for decomposers and detritivores (e.g., Richardson, 1992), an elevated turnover rate may exacerbate energy shortage from spring until autumn for the entire community.

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Supporting information of Appendix A1

Preliminary feeding activity assay with *Gammarus fossarum*

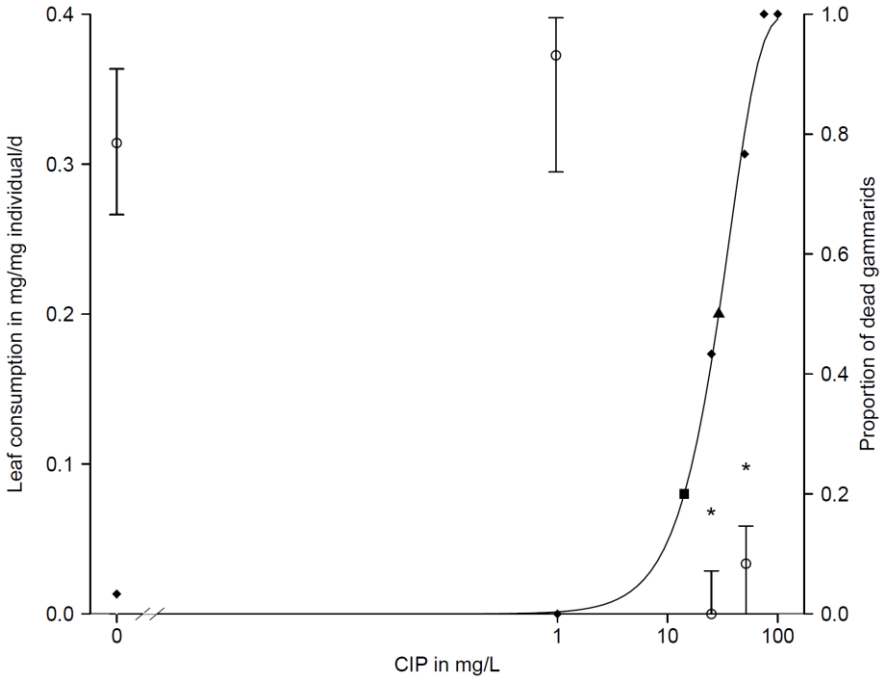

Fig. S1 Median leaf consumption (open circles with 95 % CIs; n = 30) of *G. fossarum* and proportion of dead gammarids (solid diamonds) when exposed to increasing ciprofloxacin (CIP) concentrations (0, 1, 25, 50, 75, 100 mg/L) for seven days. Moreover, the model with the best fit for mortality (see Table S2) as well as the LC_{20} and LC_{50} values (solid square = 14 mg/L [95 % CI 9.5 – 19] and triangle = 29 mg/L [95 % CI 24.5 – 34], respectively) are displayed. Asterisks indicate a statistically significant difference relative to the control. The experiments were statistically evaluated using Wilcoxon rank-sum tests (p-values were adjusted using Bonferroni's adjustment for multiple comparisons).

Multivariate evaluation of the fatty acid composition of gammarids and leaves during the long-term feeding assay

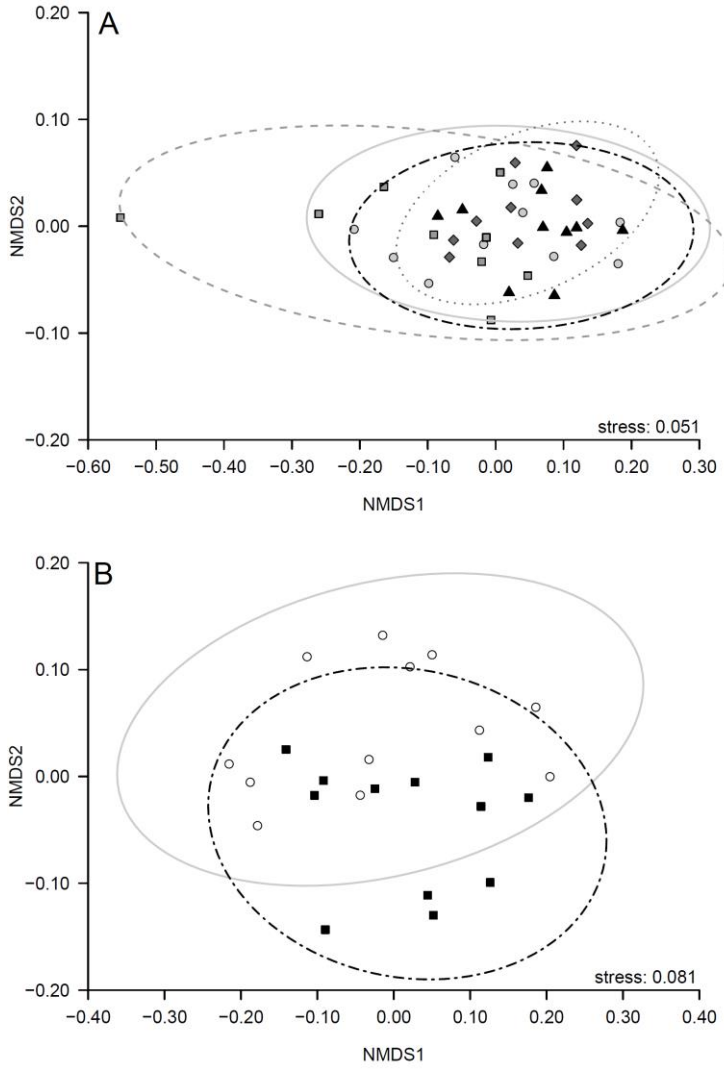

Fig. S2 Non-metric multidimensional scaling (NMDS) plots for the NLFA composition of **A** gammarids subjected to four treatments during the long-term feeding assay with 500 µg CIP/L: a CIP-free control (i.e., Control; light grey circles), gammarids being directly exposed to CIP (i.e., Water; grey squares), gammarids receiving leaves conditioned in the presence of CIP (i.e., Diet; dark grey diamonds), and a combination of both treatments (i.e., Combined; black triangles) and **B** leaf material conditioned under control conditions (white circles) and in the presence of 500 µg CIP/L (black squares) during the long-term feeding assay. Stress values are provided as a measure of "goodness-of-fit" for NMDS with reasonable fits indicated when below 0.2 (Clarke, 1993) and 95 % confidence ellipses (based on standard deviations) denote the dispersion of the data of each treatment.

Results of antibiotic analyses

Table S1 Nominal and measured (means with 95 % CIs) CIP concentrations for the respective bioassays with the respective lowest calibration level (LCL).

Assay	Test medium	Treatment	LCL (µg/L)	Nominal (µg/L)	Fresh medium (µg/L)	3-day-old medium (µg/L)
Feeding activity	SAM-5S	Control	0.50	0	<LCL	-
		CIP		500	629.0 (484.1 to 774.5)	-
				24500	25100.8 (20168.3 to 30033.4)	-
Food choice	Conditioning	Control	1.00	0	<LCL	-
		CIP		20	19.77 (5.81 to 33.72)	-
				2500	2460.7 (1945.0 to 2868.3)	-
Long-term feeding	Conditioning	Control	1.00	0	<LCL	-
		CIP		500	445.3 (417.3 to 473.3)	-
	SAM-5S	Control	1.00	0	<LCL	-
		Water		500	408.1 (320.0 to 495.2)	271.6 (255.0 to 288.3)
		Diet		-	-	18.76 (14.49 to 23.03)
		Combined		500	See Water	286.7 (264.5 to 309.0)

Concentration-response models for feeding activity data

Table S2 Models used for concentration-response modeling and their respective coefficients for each feeding activity assay.

Test	Endpoint	Model	Parameters[a]		
Preliminary experiment	Mortality	Weibull (type 2 with 2 parameters)	b=1.57	e=36807.35	-
Main experiment	Leaf consumption	Log-logistic (type 2 with 3 parameters)	b=0.559	d=0.16	e=1.854
	Mortality	Log-logistic (type 2 with 2 parameters)	b=-3.84	e=13.65	-

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

Statistical evaluations of the data of the respective bioassay

Table S3 Mortality of test organisms and group medians (with 95 % CIs) for leaf consumption for the main experiment of the two feeding activity assays (n = 30). Moreover, statistical tests used as well as p-values from statistical comparisons of CIP treatments with the control (p-values below 0.05 are printed in bold) and effect concentrations resulting in 20 and 50 % of mortality and inhibition of leaf consumption (LC_{20}/EC_{20} and LC_{50}/EC_{50} values) in mg CIP/L (with 95 % CIs) for mortality and leaf consumption are shown.

Endpoint	Concen-tration (mg/L)	Mortality (%) or Median	±95 % CI	Statistical test	p-value (after Bonferroni adjustment)	LC_{20} or EC_{20}	LC_{50} or EC_{50}
Mortality	0	6.7	0.82 to 22.07	Proportion test		9.5	13.6
	0.5	0	0.00 to 11.57		1.000	(7.6 to	(12.2 to
	6.5	6.7	0.82 to 22.07		1.000	11.4)	15.0)
	12.5	40	22.66 to 59.40		**0.003**		
	18.5	80	61.43 to 92.29		**<0.001**		
	24.5	86.7	69.28 to 96.24		**<0.001**		
Leaf consumption	0	0.16	0.14 to 0.21	Wilcoxon rank-sum		0.5	6.4
	0.5	0.13	0.10 to 0.15		0.134	(0.4 to	(5.8 to
	6.5	0.08	0.05 to 0.12		**<0.001**	0.7)	7.0)
	12.5	0.07	0.05 to 0.13		**0.002**		
	18.5	0.05	0.01 to 0.12		**<0.001**		
	24.5	0.03	0.02 to 0.05		**0.003**		

Table S4 Number of analyzed replicates and group means or medians (with 95 % CIs) for the endpoints analyzed during the food choice assay. Moreover, statistical tests used as well as *p*-values from statistical comparisons of CIP treatments with the respective control are shown. All *p*-values below 0.05 are printed in bold.

Endpoint	Concentration (µg/L)	*n*	Median	±95 % CI	Statistical test	*p*-value
Leaf consumption in mg/mg individual/d	0 (for 20)	42	0.61	0.46 to 0.78	Student's *t* (paired)	0.127
	20		0.50	0.40 to 0.62		
	0 (for 100)	43	0.41	0.12 to 0.76		0.477
	100		0.52	0.46 to 0.68		
	0 (for 500)	44	0.27	0.14 to 0.44		0.396
	500		0.32	0.15 to 0.41		
	0 (for 2500)	43	0.22	0.11 to 0.32		0.218
	2500		0.09	0.04 to 0.18		
Microbial leaf decomposition in mg/d	0 (for 20)	44	0.12	0.09 to 0.17	Wilcoxon signed-rank	1.000
	20		0.17	0.11 to 0.21		
	0 (for 100)	44	0.19	0.17 to 0.22		**<0.001**
	100		0.22	0.19 to 0.24		
	0 (for 500)	44	0.21	0.19 to 0.22		0.986
	500		0.20	0.19 to 0.23		
	0 (for 2500)	44	0.23	0.21 to 0.26		**<0.001**
	2500		0.19	0.16 to 0.22		
Fungal biomass in mg ergosterol/g leaf dry mass	0	7	0.28	0.16 to 0.36	Dunnett's *t*	
	20	7	0.31	0.15 to 0.37		0.634
	100	6	0.21	0.13 to 0.28		0.298
	500	7	0.13	0.11 to 0.19		**<0.001**
	2500	7	0.12	0.06 to 0.15		**<0.001**
Bacterial density in 10^9 cells/g leaf dry mass	0	7	0.52	0.14 to 0.88	Wilcoxon rank-sum (with Bonferroni adjustment)	
	20	7	0.22	0.11 to 0.52		0.389
	100	7	0.39	0.29 to 0.78		1.000
	500	7	0.57	0.19 to 0.81		1.000
	2500	7	0.29	0.09 to 0.55		1.000
Fungal spores/mg leaf dry mass	0	5	40.72	12.66 to 102.21	Wilcoxon rank-sum (with Bonferroni adjustment)	
	20	7	11.68	4.49 to 180.62		0.808
	100	7	6.90	4.13 to 11.57		**0.020**
	500	5	1.88	1.31 to 14.51		**0.063**
	2500	5	3.88	1.34 to 146.33		0.603
Hyphomycete community composition	0	5	_	-	PERMANOVA (with Bonferroni adjustment)	
	20	7	_	-		0.703
	100	7	_	-		**0.012**
	500	5	_	-		**0.030**
	2500	5	_	-		0.069

Table S5 Median number of sporulating fungal species per sample (with minima & maxima). SIMPER displays the contribution of spores (i.e., fungal spores/mg leaf dry mass) of each species to the dissimilarities between fungicide treatments and the respective control.

Concen-tration (µg/L)	Sporulating fungal species	SIMPER results with percentage contribution
0	3 (2 to 4)	
20	2 (2 to 4)	*F* (45); *AA* (17); *TM* (12); *NL* (11); *U* (10); *TA* (4); *CL* (0); *FF* (0)
100	2 (0 to 4)	*F* (58); *U* (11); *NL*(11); *TM* (10); *TA* (5); *AA* (5); *CL* (0); *FF* (0)
500	1 (0 to 2)	*F* (62); *NL* (10); *TM* (8); *U* (8); *FF* (7); *TA* (3); *AA* (2); *CL* (0)
2500	2 (2 to 4)	*F* (53); *TM* (18); *NL* (11); *TA* (8); *U* (7); *FF* (2); *AA* (2); *CL* (0)

Alatospora acuminata (*AA*); *Clavatospora longibrachiata* (*CL*); *Flagellospora fusarioides* (*FF*); *Fusarium* sp. (*F*); *Neonectria lugdunensis* (*NL*); *Tetracladium marchalianum* (*TM*); *Tricladium angulatum* (*TA*); Unknown (*U*)

Table S6 Number of analyzed replicates and group medians (with 95 % CIs) of parameters describing leaf quality used during the long-term feeding assay. Moreover, the statistical tests used for the respective endpoint as well as the *p*-value from the statistical comparison of 500 µg CIP/L with the control is shown. All *p*-values below 0.05 are printed in bold.

Endpoint	Concen-tration (µg/L)	*n*	Median	±95 % CI	Statistical test	*p*-value
Fungal biomass in mg ergosterol/g leaf dry mass	0	12	0.02	0.00 to 0.06	Wilcoxon rank-sum	
	500	12	0.07	0.02 to 0.12		**0.032**
Bacterial density in 10^9 cells/g leaf dry mass	0	12	0.53	0.39 to 0.91	Student's *t* (unpaired)	
	500	12	0.39	0.27 to 0.76		0.266
Total NLFA content in mg/g leaf dry mass	0	12	5.25	3.99 to 7.77	Student's *t* (unpaired)	
	500	12	4.69	3.80 to 6.40		0.399
Sat. NLFA content in mg/g leaf dry mass	0	12	1.98	1.31 to 2.92	Student's *t* (unpaired)	
	500	12	1.99	1.50 to 2.49		0.933
Monounsat. NLFA content in mg/g leaf dry mass	0	12	0.33	0.29 to 0.39	Student's *t* (unpaired)	
	500	12	0.36	0.33 to 0.44		0.339
Polyunsat. NLFA content in mg/g leaf dry mass	0	12	2.82	2.37 to 4.41	Student's *t* (unpaired)	
	500	12	2.44	1.90 to 3.18		0.183
FA composition of leaves	0	12	-	-	PERMANOVA	
	500	12	-	-		0.081

Neutral lipid fatty acid (NLFA); Saturated neutral lipid fatty acid (Sat. NLFA); Monounsaturated neutral lipid fatty acid (Monounsat. NLFA); Polyunsaturated neutral lipid fatty acid (Polyunsat. NLFA); Fatty acid composition (FA composition)

Table S7 Number of analyzed replicates and median number of fungal spores per mg dry mass of leaf material (with 95 % CIs) of each detected species during the long-term feeding assay. Since only few species and spores were detected on leaves, all medians and most of the respective confidence limits are zero. Furthermore, as the data contains too many identical values (i.e. zeros), the *p*-values originating from statistical comparisons with the respective control are not reliable and thus not reported.

Species	Concentration (µg/L)	*n*	Median	±95 % CI
Alatospora acuminata	0	12	0.00	0.00 to 0.00
	500	12	0.00	0.00 to 0.00
Mycocentrospora clavata	0	12	0.00	0.00 to 0.00
	500	12	0.00	0.00 to 0.00
Neonectria lugdunensis	0	12	0.00	0.00 to 0.00
	500	12	0.00	0.00 to 16.34

Table S8 ANOVA-tables for all gammarid-related NLFAs (n = 10) during the long-term feeding assay. All p-values <0.05 are printed in bold.

Fatty acid	Factor	df1	SS	MS	*F*-value	*p*-value
12:0	Water	1	0.2147	0.2147	3.174	0.083
	Diet	1	0.3722	0.3722	5.501	**0.025**
	Water × Diet	1	0.0548	0.0548	0.810	0.374
	Residuals	36	2.4356	0.0677		
13:0	Water	1	593	592.9	4.877	**0.034**
	Diet	1	360	360.0	2.961	0.094
	Water × Diet	1	1	0.9	0.007	0.932
	Residuals	36	4376	121.6		
14:0	Water	1	10.43	10.425	4.072	0.051
	Diet	1	10.87	10.87	4.246	**0.047**
	Water × Diet	1	4.46	4.455	1.740	0.196
	Residuals	36	92.18	2.56		
15:0	Water	1	0.0319	0.03190	1.724	0.198
	Diet	1	0.0830	0.08302	4.486	**0.041**
	Water × Diet	1	0.0055	0.0055	0.297	0.589
	Residuals	36	0.6662	0.01851		
16:0	Water	1	42.1	42.07	3.047	0.089
	Diet	1	58.8	58.8	4.259	**0.046**
	Water × Diet	1	19.6	19.6	1.419	0.241
	Residuals	36	497.1	13.81		
17:0	Water	1	0.02618	0.02618	3.608	0.066
	Diet	1	0.04691	0.04691	6.466	**0.015**
	Water × Diet	1	0.00111	0.00111	0.154	0.697
	Residuals	36	0.26119	0.00726		

Table S8 continued.

Fatty acid	Factor	df1	SS	MS	*F*-value	*p*-value
18:0	Water	1	0.534	0.5342	1.220	0.277
	Diet	1	1.165	1.1645	2.659	0.112
	Water × Diet	1	0.026	0.0265	0.060	0.807
	Residuals	36	15.765	0.4379		
14:1ω5	Water	1	0.001126	0.0011264	1.911	0.175
	Diet	1	0.001573	0.0015727	2.669	0.111
	Water × Diet	1	0.002290	0.0022901	3.886	0.056
	Residuals	36	0.021214	0.0005893		
16:1ω7	Water	1	2.728	2.7277	3.740	0.061
	Diet	1	2.311	2.3109	3.168	0.084
	Water × Diet	1	1.346	1.3455	1.845	0.183
	Residuals	36	26.259	0.7294		
18:1ω7	Water	1	0.994	0.9939	3.794	0.059
	Diet	1	0.635	0.6353	2.425	0.128
	Water × Diet	1	0.203	0.2031	0.775	0.384
	Residuals	36	9.431	0.262		
18:1ω9	Water	1	27.0	26.97	0.581	0.451
	Diet	1	131.1	131.06	2.825	0.101
	Water × Diet	1	138.5	138.48	2.985	0.093
	Residuals	36	1669.9	46.39		
20:1ω9	Water	1	0.0014	0.00139	0.037	0.849
	Diet	1	0.0595	0.05948	1.582	0.217
	Water × Diet	1	0.1426	0.14259	3.792	0.059
	Residuals	36	1.3536	0.0376		
18:2ω6	Water	1	0.10	0.100	0.030	0.863
	Diet	1	5.43	5.428	1.644	0.208
	Water × Diet	1	3.72	3.72	1.127	0.295
	Residuals	36	118.83	3.301		
18:3ω3	Water	1	13.03	13.028	1.777	0.191
	Diet	1	20.42	20.418	2.785	0.104
	Water × Diet	1	11.12	11.119	1.516	0.226
	Residuals	36	263.95	7.332		
18:3ω6	Water	1	0.000001	0.0000007	0.001	0.971
	Diet	1	0.001596	0.0015957	3.052	0.089
	Water × Diet	1	0.000001	0.0000006	0.001	0.973
	Residuals	36	0.018824	0.0005229		
20:2ω6	Water	1	0.0000	0.000011	0.001	0.981
	Diet	1	0.0139	0.013877	0.685	0.413
	Water × Diet	1	0.0268	0.026824	1.323	0.258
	Residuals	36	0.7297	0.020269		
20:3ω3	Water	1	0.0067	0.00666	0.129	0.722
	Diet	1	0.0096	0.00958	0.185	0.670
	Water × Diet	1	0.1245	0.12451	2.403	0.130
	Residuals	36	1.8651	0.05181		

Table S8 continued.

Fatty acid	Factor	df1	SS	MS	*F*-value	*p*-value
20:4ω6	Water	1	0.00007	0.000073	0.020	0.888
	Diet	1	0.00196	0.001958	0.547	0.465
	Water × Diet	1	0.00101	0.001007	0.281	0.599
	Residuals	36	0.12897	0.003582		
20:5ω3	Water	1	168	168.1	1.290	0.264
	Diet	1	462	462.4	3.548	0.068
	Water × Diet	1	8	8.1	0.062	0.805
	Residuals	36	4691	130.3		
22:6ω3	Water	1	0.000343	0.0003428	0.561	0.459
	Diet	1	0.001308	0.0013076	2.138	0.152
	Water × Diet	1	0.001236	0.0012357	2.021	0.164
	Residuals	36	0.022015	0.0006115		

Table S9 Group medians (with 95 % CIs, n = 12) of NLFAs on the leaves (mg/g dry mass of leaf material) conditioned in the absence or presence of CIP during the long-term feeding assay. Moreover, the statistical test used for the respective NLFA as well as the *p*-value from the statistical comparison of 500 µg CIP/L with the control is shown. All *p*-values below 0.05 are printed in bold.

Fatty acid	Concentration (µg/L)	Median	±95 % CI	Statistical test	*p*-value
11:0	0	0.00	0.00 to 0.00	Wilcoxon rank-sum	
	500	0.00	0.00 to 0.01		0.106
12:0	0	0.03	0.03 to 0.05	Wilcoxon rank-sum	
	500	0.02	0.02 to 0.04		**0.017**
13:0	0	0.00	0.00 to 0.00	Student's *t* (unpaired)	
	500	0.00	0.00 to 0.00		0.935
14:0	0	0.09	0.08 to 0.11	Student's *t* (unpaired)	
	500	0.08	0.06 to 0.11		0.335
15:0	0	0.01	0.01 to 0.01	Wilcoxon rank-sum	
	500	0.01	0.01 to 0.01		0.242
16:0	0	1.10	0.64 to 1.45	Student's *t* (unpaired)	
	500	0.76	0.59 to 1.19		0.085
17:0	0	0.04	0.03 to 0.06	Student's *t* (unpaired)	
	500	0.04	0.03 to 0.05		0.156
18:0	0	0.12	0.07 to 0.13	Student's *t* (unpaired)	
	500	0.11	0.09 to 0.16		0.502
20:0	0	0.19	0.14 to 0.44	Wilcoxon rank-sum	
	500	0.40	0.29 to 0.48		0.078
21:0	0	0.02	0.01 to 0.02	Wilcoxon rank-sum	
	500	0.02	0.02 to 0.03		0.143

Table S9 continued.

Fatty acid	Concentration (µg/L)	Median	±95 % CI	Statistical test	*p*-value
22:0	0	0.24	0.18 to 0.48	Student's *t* (unpaired)	
	500	0.38	0.31 to 0.49		0.113
23:0	0	0.02	0.01 to 0.02	Wilcoxon rank-sum	
	500	0.02	0.02 to 0.02		0.225
24:0	0	0.05	0.04 to 0.07	Wilcoxon rank-sum	
	500	0.07	0.05 to 0.08		0.052
14:1ω5	0	0.00	0.00 to 0.00	Wilcoxon rank-sum	
	500	0.00	0.00 to 0.00		0.101
16:1ω7	0	0.13	0.10 to 0.19	Wilcoxon rank-sum	
	500	0.18	0.15 to 0.25		0.160
18:1ω7	0	0.09	0.07 to 0.11	Student's *t* (unpaired)	
	500	0.09	0.08 to 0.10		0.851
18:1ω9	0	0.11	0.07 to 0.15	Student's *t* (unpaired)	
	500	0.08	0.07 to 0.12		0.257
20:1ω9	0	0.00	0.00 to 0.01	Wilcoxon rank-sum	
	500	0.01	0.00 to 0.01		0.590
18:2ω6	0	0.49	0.34 to 0.75	Student's *t* (unpaired)	
	500	0.37	0.27 to 0.60		0.169
18:3ω3	0	2.28	1.98 to 3.68	Student's *t* (unpaired)	
	500	1.98	1.54 to 2.55		0.169
20:2ω6	0	0.00	0.00 to 0.00	Wilcoxon rank-sum	
	500	0.00	0.00 to 0.00		0.319
20:4ω6	0	0.00	0.00 to 0.00	Wilcoxon rank-sum	
	500	0.01	0.00 to 0.01		**0.024**
22:2ω6	0	0.06	0.04 to 0.07	Student's *t* (unpaired)	
	500	0.07	0.05 to 0.11		0.060

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Appendix A2

Chronic effects of the strobilurin fungicide azoxystrobin in the leaf shredder *Gammarus fossarum* (Crustacea; Amphipoda) via two effect pathways

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Abstract

Fungicides pose a risk for crustacean leaf shredders serving as key-stone species for leaf litter breakdown in detritus-based stream ecosystems. However, little is known about the impact of strobilurin fungicides on shredders, even though they are presumed to be the most hazardous fungicide class for aquafauna. Therefore, we assessed the impact of the strobilurin azoxystrobin (AZO) on the survival, energy processing (leaf consumption and feces production), somatic growth (growth rate and molting activity), and energy reserves (neutral lipid fatty and amino acids) of the amphipod crustacean *Gammarus fossarum* via waterborne exposure and food quality-mediated (through the impact of leaf colonizing aquatic microorganisms) and thus indirect effects using 2 × 2-factorial experiments over 24 days. In a first bioassay with 30 µg AZO/L, waterborne exposure substantially reduced survival, energy processing and affected molting activity of gammarids, while no effects were observed via the dietary pathway. Furthermore, a negative growth rate (indicating a body mass loss in gammarids) was induced by waterborne exposure, which cannot be explained by a loss in neutral lipid fatty and amino acids. These energy reserves were increased indicating a disruption of the energy metabolism in *G. fossarum* caused by AZO. Contrary to the first bioassay, no waterborne AZO effects were observed during a second experiment with 15 µg AZO/L. However, an altered energy processing was determined in gammarids fed with leaves microbially colonized in the presence of AZO, which was probably caused by fungicide-induced effects on the microbial decomposition efficiency ultimately resulting in a lower food quality. The results of the present study show that diet-related strobilurin effects can occur at concentrations below those inducing waterborne toxicity. However, the latter seems to be more relevant at higher fungicide concentrations.

Keywords

Amino acids; Fatty acids; Food quality; Leaf litter breakdown; Metabolic disruptor; Microbial decomposers

Abbreviations

AA (amino acid); AZO (azoxystrobin); FA (fatty acid); FAME (Fatty acid methyl ester); NLFA (neutral lipid fatty acid); MUFA (monounsaturated fatty acid); PUFA (polyunsaturated fatty acid); SAFA (saturated fatty acid); TAG (triacylglycerol)

Introduction

Fungicides constitute the most frequently used pesticide class in European crop production (Bonanno et al., 2017) entering surface water bodies mainly via diffuse sources (e.g., surface runoff and erosion; Bereswill et al., 2012). Consequently, fungicides are frequently detected in surface waters (e.g. Stenrød, 2015; Schreiner et al., 2016). Owing to their modes of action that target evolutionarily conserved molecular processes (Stenersen, 2004), fungicides can impair ecosystem structure and functioning in exposed aquatic ecosystems (Fernández et al., 2015).

Fungicides have, for example, been reported to affect heterotrophic processes in aquatic systems involving aquatic fungi and detritivorous macroinvertebrates (i.e., shredders; e.g., Artigas et al., 2012; Zubrod et al., 2014). Fungi, in particular aquatic hyphomycetes (i.e., a polyphyletic mitosporic group of fungi; Baschien et al., 2006), integrate carbon and energy into aquatic food webs by degrading leaf litter (Hieber and Gessner, 2002; Baldy et al., 2007). At the same time, they increase the nutritional quality of leaf litter for shredders (i.e., microbial conditioning; e.g., Bärlocher and Kendrick, 1975b; Graça et al., 1993). The fine particulate organic matter ultimately produced by leaf shredding invertebrates (i.e., feces) serves as food source for collectors (Bundschuh and McKie, 2016). Moreover, shredders are an important prey, for instance, for fish (MacNeil et al., 1999). Waterborne fungicide exposure at environmentally relevant concentrations can induce detrimental effects in shredders (e.g., Kunz et al., 2017; Zubrod et al., 2017). Fungicides may also interfere with aquatic fungi colonizing leaves ultimately changing the quality of leaves as food for shredders, impacting their physiology (i.e., dietary pathway; sensu Zubrod et al., 2015c).

Amongst fungicide classes, strobilurins are of particular concern for aquatic invertebrates: firstly, they dominate, along with triazole fungicides, the global fungicide sales (Oliver and Hewitt, 2014), indicating intensive use and likely frequent exposure of aquatic ecosystems (see for detailed description of physical-chemical properties and exposure Zubrod et al., 2019). Secondly, due to their mode of action (inhibition of the mitochondrial respiratory chain; Bartlett et al., 2002), strobilurins are highly toxic to a broad range of non-target organisms and, hence, pose a high risk for aquatic ecosystems qualifying strobilurins as one of the most hazardous fungicide classes (Zubrod et al., 2019). Despite these risks, knowledge on waterborne and dietary effect pathways of strobilurins in heterotrophic systems is scarce.

To address this knowledge gap, we assessed survival and sublethal effects on the amphipod *Gammarus fossarum* over 24 days of exposure to the model strobilurin azoxystrobin (AZO) through waterborne and dietary pathways and their combination using a 2 × 2 factorial design. *G. fossarum* was selected as model shredder, since this species constitutes a key leaf-shredder in low-order streams of the Northern Hemisphere (Piscart et al., 2009). Furthermore, *Gammarus* spp. are valuable test organisms for the effect assessment of pollutants, because they are highly susceptible to chemical stressors, various ecotoxicologically relevant endpoints (physiological and behavioral responses) can be quantified and they are easy to handle in the laboratory (Kunz et al., 2010). As chemical stress can disturb the energy balance of invertebrates (Sokolova et al., 2012), energy processing (leaf consumption and feces production), somatic growth (measured by growth rate and molting activity), and energy reserves (fatty and amino acids, i.e., FAs and AAs) of *G. fossarum* were quantified. To document AZO-induced alterations of the microorganism-mediated food quality for gammarids, ultimately resulting in changes in assimilation (indicated by the difference in food intake und excretion) and somatic growth (Zubrod et al., 2015c), fungal biomass, bacterial density and FAs of leaves were analyzed.

We expected that waterborne AZO exposure would cause higher effects on energy processing, somatic growth, and energy reserves (i.e., FAs and AAs) of gammarids compared to exposure via the dietary pathway. This hypothesis is based on previous studies suggesting that waterborne fungicide exposure triggers detoxification mechanisms, ultimately disrupting the organisms' energy homeostasis. Fungicide-induced changes in food quality (diet-related fungicide effects), in contrast, affect the food assimilation of shredders but do not induce energy-intensive stress responses (Zubrod et al., 2015c; Feckler et al., 2016). When both pathways act jointly, we expected additive actions (i.e., no interaction effects) for all measured endpoints as observed for an organic fungicide mixture (cf. Zubrod et al., 2015c). We present and discuss our results separated for the effects of the two experiments employing different concentrations of the fungicide azoxystrobin.

Material and methods

Experimental design and test substance

Bioassays were performed in July and November 2016 with 30 and 15 µg AZO/L, respectively, and followed established protocols for examination of long-term pesticide effects in gammarids over 24 days (i.e., with control mortality < 20 %) under laboratory conditions (cf. Zubrod et al., 2015b; Zubrod et al., 2015c). The factorial design consisted of four treatments where the amphipod was either subjected to an AZO-free control, waterborne AZO exposure (i.e., Water), leaves microbially colonized in the presence of AZO (i.e., Diet) or a combination of both effect pathways (i.e., Combined; see Konschak et al., 2020 for a schematic representation of the test design). The first bioassay, which assessed 30 µg AZO/L, was performed with 48 replicates per treatment. This concentration was selected as it induces adverse effects in aquatic invertebrates (Zafar et al., 2012; van Wijngaarden et al., 2014) and these levels have been reported in European surface waters (see Berenzen et al., 2005). Due to high mortality during the first bioassay, a second bioassay was performed with a lower concentration (i.e., 15 µg/L) and a higher replication (n = 60) examining sublethal effects in gammarids. To obtain the respective AZO concentration, stock solutions were prepared by diluting the commercially available product Ortiva (Syngenta Agro GmbH, Basel, Switzerland) in the respective test medium (see below), which made the use of further solvents redundant.

Sources of leaves, microorganisms and gammarids

In October 2015, leaves of black alder (*Alnus glutinosa*) were collected before abscission from trees near Landau, Germany (49°20´N; 8°09´E) and stored at −20 °C. Before starting the microbial conditioning of leaves serving as food during each bioassay, leaves were defrosted and filled in mesh bags (mesh size ~1 mm). They were subsequently deployed for 2 weeks in the stream Rodenbach, Germany (49°33´N; 8°02´E) upstream of agricultural activities and settlements to obtain a substrate with a near-natural microbial community. In the laboratory, the leaves were mixed with uncolonized black alder leaves in a stainless-steel container filled with 30 L of a nutrient medium (i.e., conditioning medium; Dang et al., 2005). Leaves were kept at 16 ± 1 °C under continuous aeration and in total darkness (i.e., laboratory conditions). After 2 weeks, they were used as microbial inoculum for leaf conditioning.

As per Zubrod et al. (2010), gammarids were collected in the stream Hainbach, Germany (49°14´N; 8°03´E) upstream of agricultural actives and settlements, 1 week before starting each bioassay and subsequently separated into different size classes in the laboratory (cf. Franke, 1977). To reduce the within-treatment variability, only males (identified by its precopula position) with no acanthocephalan infestation (identified by red spots in the pereon or pleon) and a cephalothorax length of 1.2 – 1.6 mm were used during the bioassay with 30 µg AZO/L. However, both males and females had to be used for the second bioassay in November 2016 since gammarids were not found in precopula pairs due to their reproductive resting period (Becker et al., 2013). In the laboratory, animals were fed *ad libitum* with conditioned black alder leaves and stepwise acclimatized to a culture medium (SAM-5S, Borgmann, 1996) over 7 days.

Long-term feeding bioassay

Since the present study followed established protocols, the experimental setup is reported in detail elsewhere and therefore only briefly described here (Zubrod et al., 2015b; Zubrod et al., 2015c). For each bioassay, leaf strips were cut from uncolonized black alder leaves. Leaf strips were subsequently conditioned in 15-L aquaria filled with 12 L conditioning medium and 50 – 60 g (wet weight) of the microbial inoculum (see above) for 12 days under laboratory conditions in the absence or presence of the respective AZO concentration. Four independent leaf conditioning phases were started at 6-day intervals, to guarantee *ad libitum* feeding with freshly conditioned food over the entire experimental period. For the first bioassay, two aquaria with 180 leaf strips and 60 g (wet weight) microbial inoculum each per treatment were used for every conditioning phase. During the second bioassay, the higher number of replicates required a higher amount of food, leading to three aquaria with 150 leaf strips and 50 g microbial inoculum each. In each conditioning phase, the medium (with the respective fungicide concentration) was renewed every 3 days to ensure a chronic fungicide exposure. After 12 days, three pairs of two leaf discs originating from three different leaf strips were cut and introduced into the bioassay. Additionally, leaf strips and discs were preserved for fatty acid and microbial analyses, respectively.

Each replicate consisted of a 250-mL glass beaker containing 200 mL of SAM-5S, a cylindrical and rectangular mesh cage made from stainless steel mesh screen (mesh size = 0.5 mm). One gammarid and three leaf discs originating from three different strips were kept in the cylindrical cage. The remaining three leaf discs from the same three leaf strips were deployed in the rectangular stainless-steel mesh cage and were used to determine microbial

and handling-related leaf mass losses. A watch glass protected the leaf discs in the rectangular cage against potential interactions with the feces of gammarids (see Zubrod et al., 2015b for a schematic representation of a replicate). Every 3 days, SAM-5S with the respective AZO concentration were renewed. At the same time, molting and dead organisms were recorded and removed from the test. The 3-day old SAM-5S was filtered through a pre-weighed glass fiber filter (GF/6, Whatman, Dassel, Germany) to quantify feces produced by *G. fossarum* (see for handling details of the filters Zubrod et al., 2015b). Every 6 days, gammarids were provided with freshly conditioned leaf discs and the leaf disc remains were removed. Leaf disc remains as well as filters were dried at 60 °C and weighed to the nearest 0.01 mg. At the end of the bioassay, surviving animals were shock-frozen using liquid nitrogen and stored at −80 °C before being lyophilized and weighed to the nearest 0.01 mg.

Microbial analyses

Ergosterol, a proxy for fungal biomass (Gessner, 2005), and bacterial density were analyzed. After each microbial leaf conditioning phase, five leaf strips of each aquarium (in total 24 and 35 samples, respectively) were stored at −20 °C for ergosterol analysis and three leaf discs per replicate (Ø = 16 mm) were stored at 4 °C (in a 2 % formaldehyde/0.1 % sodium pyrophosphate solution) for the quantification of bacterial densities.

As per Gessner and Schmitt (1996), ergosterol was separated and concentrated using solid-phase extraction (Sep-Pak® Vac RC tC18 500 mg sorbent, Waters, Milford, US-MA). Extracts were measured by high-performance liquid chromatography with UV–visible detection (1200 Series, Agilent Technologies, Santa Clara, US-CA) using a LiChrospher® 100 RP-18 column (250 mm × 4.6 mm, particle size 5 µm, Merck Millipore, Billerica, US-MA). Ergosterol concentrations were determined via external standard calibration and normalized to leaf dry mass.

Following Buesing (2005), bacteria cells were detached from the leaf discs via ultrasonication and stained by SYBR® Green II (Molecular Probes, Eugene, US-OR). Cell numbers were determined by taking 20 digital photographs via a fluorescence microscope in combination with the software AxioVision (Axio Scope.A1, AxioCam MRm and AxioVision Rel. 4.8, Carl Zeiss MicroImaging, Jena, Germany) and extrapolating the number of cells to the total sample volume. Cell numbers were normalized to leaf dry mass of three additional leaf discs per sample from the same leaf strips, which were dried at 60 °C and weighed to the nearest 0.01 mg.

Fatty acid analyses

Triacylglycerol (TAG) fatty acids (i.e., neutral lipid fatty acids, NLFAs), which represent the major energy reserves in invertebrates (Azeez et al., 2014), were analyzed to shed light on implications of *Gammarus* physiology. Therefore, six and ten gammarids per treatment for the first and second bioassay, respectively, were lyophilized and weighed as described above. According to Konschak et al. (2020), gammarids were homogenized, a TAG with deuterated 18:0 FAs (Tristearin-D105, Larodan, Solna, Sweden) serving as internal standard was added and the homogenate was stored in a chloroform/methanol/water mixture overnight at 4 °C. TAGs were separated and concentrated by eluting 4 ml of chloroform through a solid phase extraction column (Chromabond® easy polypropylene columns, Macherey-Nagel, Düren, Germany) and NLFAs were transesterified using trimethylsulfonium hydroxide (Sigma-Aldrich, St. Louis, US-MO; for more details, see Konschak et al., 2020). Fatty acid methyl esters (FAMEs) were determined via gas chromatography (GC; CP-3800, Varian, Palo Alto, US-CA) with flame ionization detector (FID), a DB-225 GC column (30 m, ID 0.25 mm, film thickness 0.25 µm, J&W Scientific, Agilent Technologies, Santa Clara, US-CA; cf. Fink, 2013) and nitrogen as carrier gas. FAMEs were identified using retention times of standards (Sigma-Aldrich, St. Louis, US-MO). NLFA concentrations in µg/mL were determined via external standard calibration, blank correction and the recovery rate of the internal standard. Corrected NLFA concentrations were extrapolated to the total sample and normalized to dry mass of the gammarid (i.e., µg/mg).

Since effects on the energy processing of gammarids via the dietary pathway were observed at 15 µg AZO/L, total FAs of leaves (40 mg leaf dry mass per aquarium; in total 24 samples) were analyzed as proxy for alterations in the microbial conditioning process (Torres-Ruiz and Wehr, 2010), indicating changes in the food quality (Zubrod et al., 2015a). Therefore, leaves were lyophilized, manually crushed and weighed as described above. The derivatization of FAs to FAMEs via 3N methanolic HCl (Sigma-Aldrich, St. Louis, US-MO) and the subsequent liquid-liquid extraction of FAMEs using isohexane was performed according to Fink (2013), since the method improved the purification of the analyzed samples compared to the rapid transesterification of FAs to FAMEs with trimethylsulfonium hydroxide. FAMEs were analyzed and FAs in µg/mg leaf dry mass were quantified as described above.

Amino acid analyses

Amino acids of gammarids subjected to 30 µg AZO/L were analyzed as, alongside TAGs, proteins constitute an important energy source for gammarids (Hervant et al., 1999). Prior to analyzing AAs via GC-FID, six gammarids per treatment (in total 24 samples) were lyophilized, weighed as described above and manually crushed with a glass pipette. Proteins of gammarids were hydrolyzed to free AAs using 1 mL of 6 N HCl for 24 hours at 110 °C. Afterwards, AAs were quantified via the EZ:Faast™ kit (Amino Acid Analysis of Protein Hydrolysates by GC-FID or GC-NPD, Phenomenex Inc., Torrance, US-CA) as described in detail by Badawy (2019). Briefly, a 100 µL aliquot of sample was diluted in a sodium carbonate washing solution and, subsequently, 25 µL of the dilution was added to 100 µL of an internal standard (norvaline). AAs were concentrated and purified via solid-phase extraction followed by AA derivatization using a reagent containing propyl chloroformate. AA derivatives were extracted by using a liquid-liquid extraction step and subsequently measured via GC-FID and a Zebron™ ZB-AAA GC column (component of the EZ:Faast™ kit). Nitrogen was used as carrier gas. AAs were identified and quantified (i.e., using external calibration) via an AA standard mixture, extrapolated to the total sample and normalized to dry mass of the gammarids.

Fungicide analyses

Nominal AZO concentrations were verified by taking samples from each 15-L aquarium at the start of the conditioning and after three days, and by randomly sampling from four replicates per bioassay treatment at day 0, 3, 6, 9, 12, 15, 18 and 21. Samples were preserved at −20 °C until analysis. AZO concentrations were determined via ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS; Thermo Fisher Scientific, Bremen, Germany) and external matrix-aligned standard calibration (for more details see Zubrod et al., 2015c). Since mean measured AZO concentrations did deviate only slightly more than 20 % (up to +20.2 %) in one case from the nominal concentrations (Table S1), nominal concentrations are reported throughout this manuscript.

Calculations and statistics

The leaf consumption of gammarids and feces production (both in mg/day) were calculated as described by Zubrod et al. (2011). The growth rate of *G. fossarum* in µg dry mass gain/day was calculated by subtracting the mean dry mass of 21 and 54 lyophilized gammarids, respectively, at the start of the bioassay with 30 and 15 µg AZO/L from the final dry mass of

each animal divided by 24 days. (NL)FAs and AAs were assessed individually as well as in sum as proxy for saturated FAs (SAFAs), monounsaturated FAs (MUFAs), polyunsaturated FAs (PUFAs) and total AAs, respectively.

Data were visually checked for extreme values by using boxplots with a 1.5 × interquartile range. Consequently, one data point of the feces production and NLFA data set, respectively, was removed each from further analyses. Normality and homoscedasticity of data were tested via Shapiro–Wilk and Levene's test, respectively, as well as using visual inspection.

Two-level hierarchical data (i.e. repeated measures of the leaf consumption and feces production are nested within individual gammarids) were evaluated via multilevel analysis. The full model consisted of the continuous predictor variable *time*, the two-level factorial predictor variable *Water* and *Diet* and their interaction (*Water* × *Diet*) as fixed effects. Furthermore, individual intercepts constituted the random part of the model. To test the effect of each factorial predictor variable on the respective response variable, factorial predictor variables were added stepwise to the model and compared with a model without this variable (see Table 1). The statistically significant effect of each factorial predictor variable was finally evaluated using a likelihood ratio test (Field et al., 2012). Prior to modeling, non-normally distributed data were log-transformed or rank-transformed. Parametric and non-parametric data with two factors and two factor levels (i.e., growth rate as well as NLFAs and AAs of gammarids) were analyzed via two-way ANOVA and rank transformed two-way ANOVA, respectively. Parametric and non-parametric data with one factor and two levels (i.e., microbial parameters) were evaluated using Student's *t*-test and Wilcoxon rank-sum test, respectively. Analysis of mortality and molting data were conducted via binary logistic regression (Field et al., 2012) and Kaplan–Meier estimation was performed for further survival analysis (e.g., Jager et al., 2008). Multivariate data (i.e., FA and AA composition) were square-root transformed to reduce the discriminatory power of dominant NLFAs and AAs (Happel et al., 2017) and analyzed using permutational multivariate analysis of variance (PERMANOVA). Detailed information on statistical tests and outcomes (e.g., *p*-values, *F*-statistics, medians with 95 % confidence intervals) are provided in Tables 1 – 3 and S2 – S9. Modeling, statistics and figures were performed using R Version 3.5.1 for Windows (R Core Team, 2014) and the add-on packages *asbio*, *car*, *drc*, *nlme*, *plotrix*, *survival*, and *vegan*. The term "significant" denotes statistical significance at the level of 0.05 through the whole study.

Table 1 Results of likelihood ratio (χ^2) tests comparing multilevel models by stepwise adding factorial predictor variables (i.e., *Water*, *Diet* and *Water* × *Diet*) for the respective endpoint of each bioassay (see text for model details). ΔAIC values represent the alteration in model fit to the previous model (negative values indicate an improvement), *p*-values printed in bold indicate a statistically significant impact of the predictor variable on the respective endpoint and Est. (with ±95 % CI) and SE representing the parameter estimate (indicating the predictor variable's effect direction) and its standard error, respectively.

Concen-tration (µg/L)	Endpoint	Added factorial predictor	Model	ΔAIC	Compared to model	χ^2	*p*-value	Est. (±95 % CI)	SE
30	Leaf consumption	-	1						
		Water	2	-33.05	1	35.046	**<0.001**	-0.23 (±0.08)	0.04
		Water + *Diet*	3	1.82	2	0.180	0.671		
		Water + *Diet* + *Water* × *Diet*	4	1.31	3	0.686	0.408		
	Feces production	-	1						
	(rank transformed)	*Water*	2	-56.84	1	58.844	**<0.001**	-129.82 (±31.21)	15.85
		Water + *Diet*	3	1.84	2	0.160	0.689		
		Water + *Diet* + *Water* × *Diet*	4	1.47	3	0.530	0.467		
15	Leaf consumption	-	1						
		Water	2	-0.78	1	2.783	0.095		
		Water + *Diet*	3	1.40	2	0.603	0.438		
		Water + *Diet* + *Water* × *Diet*	4	1.17	3	0.831	0.362		
	Feces production	-	1						
	(log-transformed)	*Water*	2	0.86	1	1.137	0.286		
		Water + *Diet*	3	-6.49	2	8.492	**0.004**	0.028 (±0.02)	0.01
		Water + *Diet* + *Water* × *Diet*	4	1.17	3	0.828	0.363		

Results and discussion

24-day bioassay with 30 µg AZO/L

In accordance with our hypothesis, waterborne AZO exposure (30 µg/L) caused higher effects compared to the dietary pathway. In fact, waterborne exposure resulted in 50 % (significant) mortality over the study duration, while this variable remained on the control level in the dietary treatment (Fig. 1; Table S2, S3). This high mortality after 24 days of exposure was observed at an AZO concentration roughly 9-fold and 5-fold higher for *G. pulex* and *G. fossarum* after 4 and 7 days of exposure, respectively (Beketov and Liess, 2008; Zubrod et al., 2014). Moreover, the observed high chronic toxicity is in line with a recent study using

another amphipod shredder, *Hyalella azteca*, reporting 50 % mortality at 9.5 µg/L after up to 42 days of exposure (Kunz et al., 2017). Although the mode of action of AZO in aquatic invertebrates is unknown, it is suggested that the inhibition of mitochondrial respiration induces cellular oxidative stress (Elskus, 2012; Rodrigues et al., 2013), damaging essential cellular biomolecules (e.g., DNA, proteins and lipids) and ultimately causing cell death (Lushchak, 2011). Furthermore, the high mortality could be related to a synergistic effect of the fungicide stress and increased reproductive activities of male gammarids (prior to field sampling) in spring / summer. Gammarids could be more susceptible to AZO in spring / summer than in late autumn / early winter, due to higher energy expenditures for other energy requiring functions (e.g., mating activity; Becker et al., 2013) reducing the energy investment in maintenance (stress protection and damage repair). The contribution of energy investment in reproduction to the observed toxicity cannot be determined here but could stimulate future research.

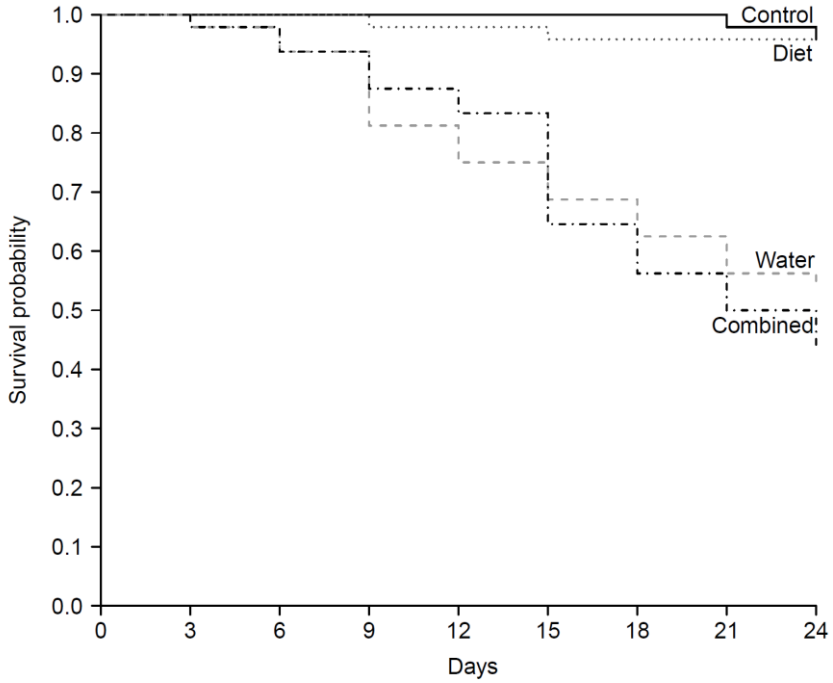

Fig. 1 Kaplan-Meier survival plot for *G. fossarum* subjected to the Control (black solid line), Water (light grey dashed line), Diet (dark grey dotted line) and Combined (black dot-dashed line) treatment during the 24-day bioassay with 30 µg AZO/L (see for Kaplan-Meier estimates Table S3). Statistical analyses are displayed in Table S2.

Besides lethal effects, molting activity of surviving gammarids was increased under waterborne AZO exposure (Fig. 2; Table S2). This response could be caused by an AZO-induced increased activity of molting hormones (hydroxylated ecdysteroids; Zou, 2020),

which, however, needs further attention in follow up experiments. Moreover, waterborne AZO exposure reduced gammarids' leaf consumption (up to 65 %; Fig. 3a), resulting in a significantly lower feces production (up to 70 %; Fig. 3b; Table 1, 2). Over the course of the study, leaf consumption and feces production increased in the Water and Combined treatment approaching the control level. This recovery is most likely explained by variability in tolerance to chemical stress among individuals within the same population (Barata et al., 2002). Probably, the most sensitive gammarids (i.e., those whose leaf consumption and feces production were most affected) died during the bioassay leaving more tolerant specimen behind. However, a significantly negative growth rate (i.e., animals lost body mass) was observed for surviving animals at test termination (Fig. 4a; Table 2). Therefore, it seems plausible that the mass loss (~10 %) was caused by a reduction of TAGs or proteins due to an increased use of energy reserves as, in fact, AZO increases the energy expenditure for defense and repair mechanisms in animals by inducing oxidative stress (Han et al., 2014; Han et al., 2016). Contrary to this assumption, NLFAs and AAs were non-significantly and significantly elevated (Tables 2, and S4 – S7), respectively, indicating that AZO induced a metabolic dysfunction. Indeed, chemical stressors can disrupt energy metabolism in invertebrates and, thus, organisms' energy homeostasis (Lee et al., 2018). The mitochondrial oxidative phosphorylation inhibitor AZO, for instance, acts as metabolic disruptor by impairing mitochondrial respiration and thus promotes TAG accumulation in the organism (Kassotis and Stapleton, 2019) as well as possibly inhibited the catabolism of AAs. Although, stress-induced shifts in AA composition were also shown in previous studies (e.g., Powell et al., 1982; Graney and Giesy, 1987), this variable has hardly been studied. As a consequence of this insufficient knowledge of biochemical processes, these data are challenging to interpret and only descriptively presented in the present work. Nonetheless, the negative growth rate could be explained by the depletion of other biomolecules, such as the carbohydrate glycogen, utilized to cope with energy demands to maintain vital processes in organisms (Willmer et al., 2005). Contrary to FAs, glucose first undergoes the anaerobic metabolic pathway (i.e., glycolysis) for energy production and thus does not rely completely on the aerobic metabolic pathway (i.e., Krebs cycle followed by the oxidative phosphorylation; Sokolova et al., 2012), which is potentially disrupted by AZO. This assumption finds support in the gammarid weight loss of ~10 % detected during the present study, an effect size that equals the reported glycogen concentration in *G. fossarum* (Koop et al., 2011). Hence, gammarids may have exhausted their glycogen reserves under waterborne AZO exposure. Even though some AAs

(e.g., aspartate and glutamate) can be metabolized via the anaerobic pathway in crustaceans, their utilization for energy production is comparably low (Hervant et al., 1995).

Table 2 ANOVA-tables for all gammarid-related endpoints during the long-term feeding assay with 30 µg AZO/L. All *p*-values <0.05 are printed in bold.

Endpoint	Factorial predictor	df1	SS	MS/R2	*F*-value	*p*-value	ANOVA type
Growth rate	*Water*	1	45016	-	37.765	**<0.001**	*Two-way
	Diet	1	1702	-	1.428	0.234	ANOVA
	Water × Diet	1	6192	-	5.195	**0.024**	(rank trans-
	Residuals	132	157344	-			formed)
SAFA content	*Water*	1	10.4	10.438	0.373	0.548	Two-way
	Diet	1	3.3	3.305	0.118	0.735	ANOVA
	Water × Diet	1	15.2	15.154	0.542	0.471	
	Residuals	19	531.2	27.959			
MUFA content	*Water*	1	16.0	16.02	0.218	0.646	Two-way
	Diet	1	1.6	1.56	0.021	0.886	ANOVA
	Water × Diet	1	50.3	50.33	0.685	0.418	
	Residuals	19	1395.9	73.47			
PUFA content	*Water*	1	3.39	3.391	0.256	0.618	Two-way
	Diet	1	4.15	4.151	0.314	0.582	ANOVA
	Water × Diet	1	0.65	0.649	0.049	0.827	
	Residuals	19	251.28	13.225			
NLFA compo-	*Water*	1	0.002421	0.01090	0.219	0.821	PERMANOVA
sition of	*Diet*	1	0.002425	0.01092	0.219	0.809	(square root
gammarids	*Water × Diet*	1	0.007054	0.03176	0.638	0.478	transformed)
	Residuals	19	0.210231	0.94643			
AA content	*Water*	1	12775	12775	8.917	**0.007**	Two-way
	Diet	1	82	82	0.057	0.814	ANOVA
	Water × Diet	1	3973	3973	2.773	0.111	
	Residuals	20	28652	1433			
AA composition	*Water*	1	0.008694	0.27123	8.565	**0.009**	PERMANOVA
of gammarids	*Diet*	1	0.000218	0.00679	0.214	0.711	(square root
	Water × Diet	1	0.002842	0.08866	2.800	0.095	transformed)
	Residuals	20	0.020301	0.63332			

*Due to unbalanced data, Type II instead of Type I sums of squares were used (Langsrud, 2003)

As expected, when the dietary and waterborne pathway act jointly, additive actions were observed for the energy processing of gammarids (Table 1), while an interaction effect of both pathways was detected for the growth rate (Table 2). Antagonism was concluded for this endpoint, as the observed growth rate reduction of 210 % in the Combined treatment relative to the control cannot be explained by adding up the effect sizes of the sole pathways

(i.e. expected effect size is ~330 % based on 80 % and 250 % reduction in the Diet and Water treatment). It is possible that the depletion of carbohydrates reached almost its maximum level in the presence of waterborne AZO exposure during the 24-day bioassay. This might explain the similar median reduction of the growth rate in the Water (~250 %) and the Combined treatment (~210 %), which in turn supports the hypothesis that AZO via the dietary pathway seems to be of minor importance.

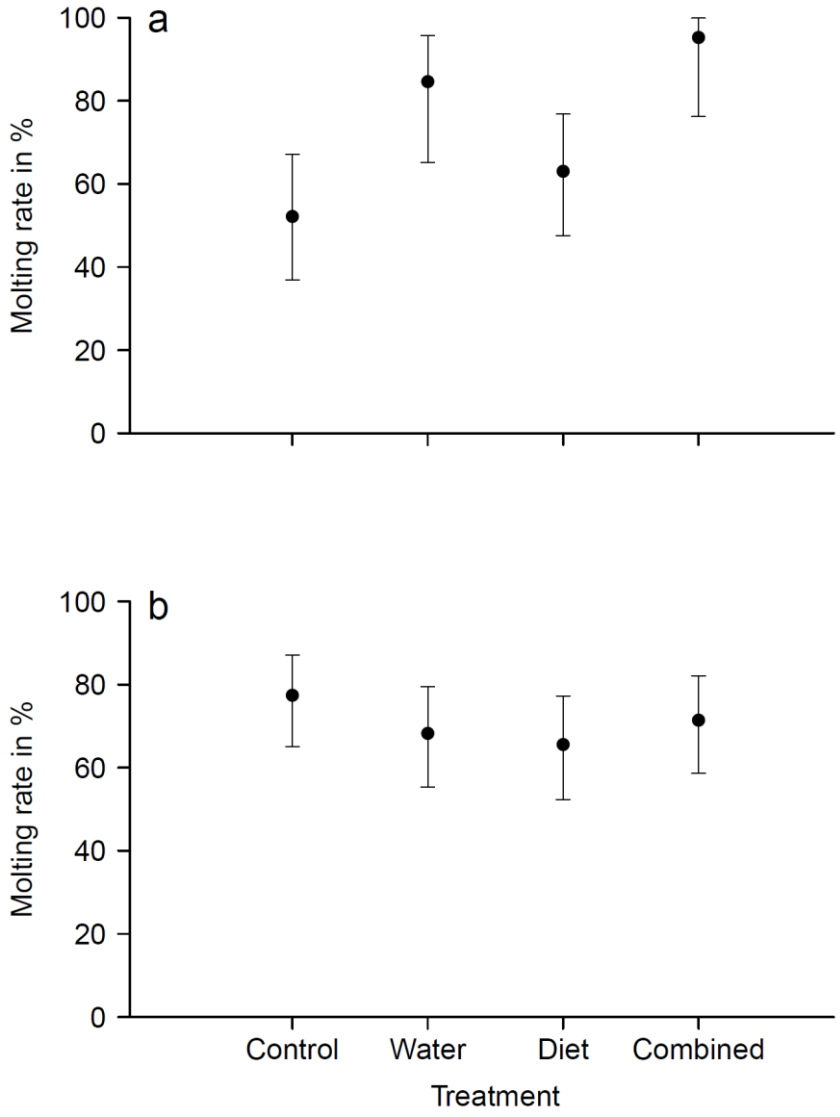

Fig. 2 Rate of molted individuals of surviving gammarids (with 95 % CIs) subjected to different effect pathways during the 24-day bioassays with 30 (a) and 15 (b) µg AZO/L. Statistical analyses are displayed in Table S2.

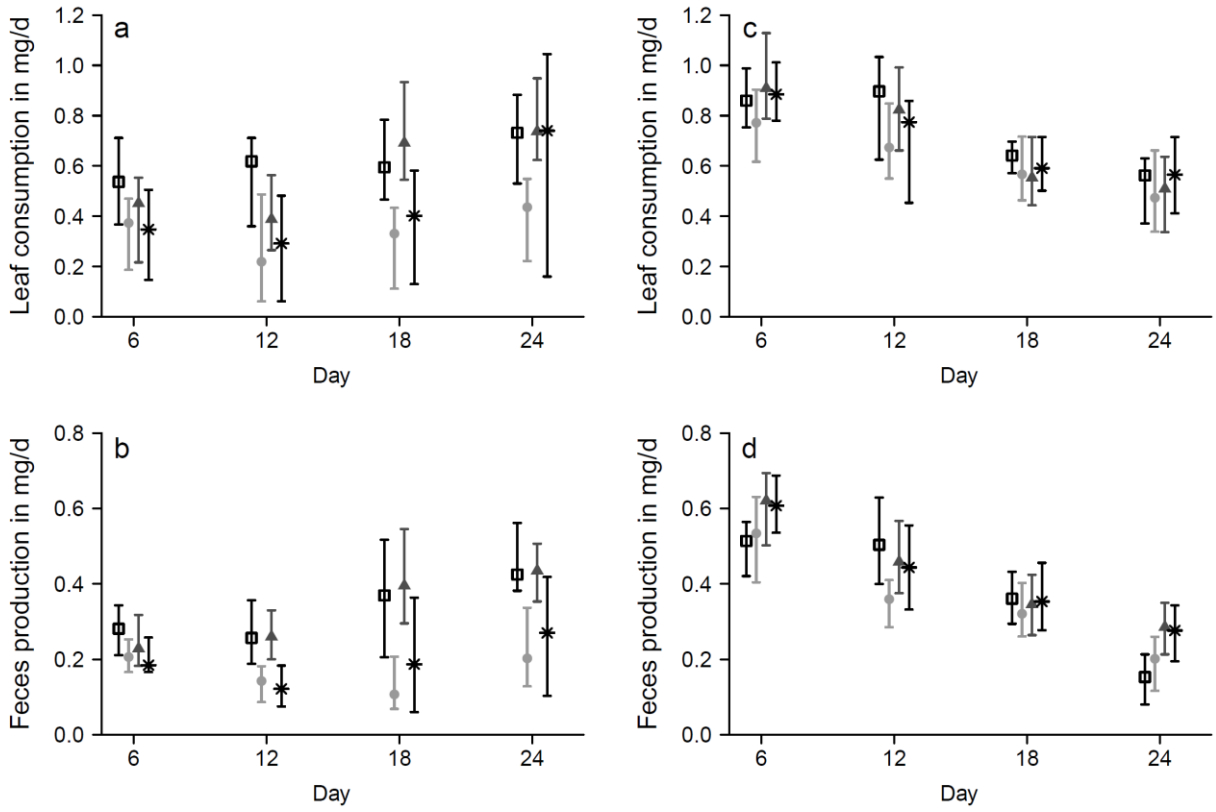

Fig. 3 Median (with 95 % CIs) leaf consumption and feces production of *G. fossarum* subjected to the Control (black square), Water (light grey dot), Diet (dark grey triangle) and Combined (black asterisk) treatments during the 24-day bioassays with 30 (a and b) and 15 (c and d) µg AZO/L. Statistical analyses are displayed in Table 1.

24-day bioassay with 15 µg AZO/L

Contrary to our expectations, 15 µg AZO/L did not affect the energy processing and physiology of gammarids via the waterborne pathway (Fig. 2b, 3c, 3d, 4b; Tables 1, 3). Zubrod et al. (2014) suggested that AZO has a steep dose-response relationship, which may indicate that 15 µg AZO/L is below the threshold concentration causing effects on the measured response variables in gammarids during 24 days. However, seasonal variations in sensitivity of *Gammarus* ssp. to waterborne exposure of chemical stressors complicates a direct comparison of both bioassays (cf. Dalhoff et al., 2018), even though animals with similar size (i.e., cephalothorax length of 1.2 – 1.6 mm) acclimatized under laboratory conditions prior to the start of each bioassay were used. Moreover, it is possible that the involvement of both sexes during the second bioassay masked adverse effects by increasing variability in the assessed population as male and female gammarids differ in their physiology and, thus, probably in their sensitivity towards contaminants (e.g., Gismondi et al., 2012).

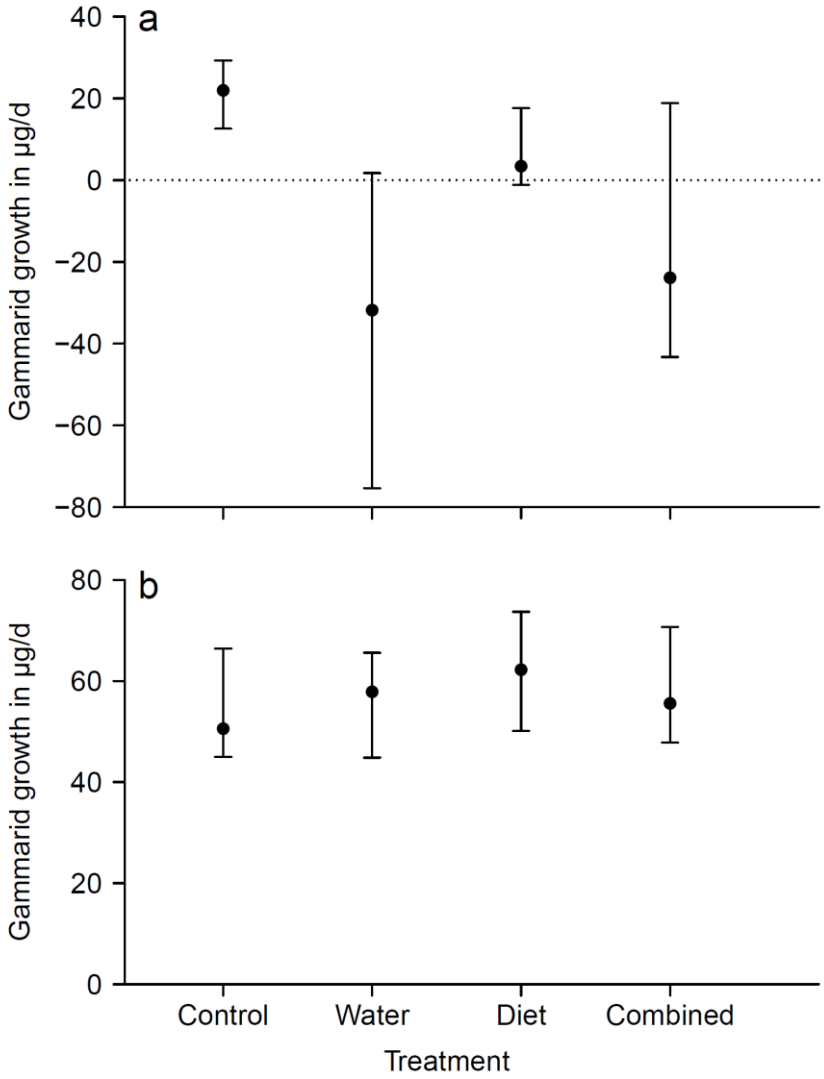

Fig. 4 Median (with 95 % CIs) growth rate of *G. fossarum* subjected to different effect pathways during the 24-day bioassays with 30 (a) and 15 (b) µg AZO/L. Statistical analyses are displayed in Table 2.

When *G. fossarum* was fed leaves conditioned in the presence of 15 µg AZO/L, leaf consumption and physiological fitness (i.e., somatic growth and energy reserves) were not significantly affected (Table 1, 3, S2), but feces production was significantly increased (up to 85 %; Fig. 3d; Table 1), suggesting a lower assimilation efficiency likely triggered by a lower food quality (Bärlocher and Kendrick, 1975a). Indeed, FA contents of leaves conditioned in presence of AZO were generally higher (Tables S8, S9). As the FA content decreases during leaf litter breakdown these higher FA levels indicate a lower microbial activity (Torres-Ruiz and Wehr, 2010). However, an AZO-induced decrease in ergosterol content (i.e., proxy for fungal biomass) and bacterial densities was not observed, suggesting AZO to trigger direct or indirect (through changes in microbial community structure) reductions in decomposition

efficiency. Comparable effects were not observed in the first bioassay, which is most likely explained by the use of microbial inocula from different seasons (summer vs. autumn) resulting in differences in the leaf-associated fungal species composition (Nikolcheva and Bärlocher, 2005) with a potentially different susceptibility to fungicide stress (cf. Zubrod et al., 2015a). Taken together, our data suggest that AZO can affect shredders' energy processing indirectly (via the dietary pathway) at concentrations not considered harmful when applied via the water phase. This indirect pathway is likely triggered by alterations in leaves' microbial conditioning and, consequently, food quality. The mechanisms are, however, not yet fully understood.

Table 3 ANOVA-tables for all gammarid-related endpoints during the long-term feeding assay with 15 µg AZO/L.

Endpoint	Factorial predictor	df1	SS	MS/R2	*F*-value	*p*-value	ANOVA type
Growth rate	*Water*	1	439	439	0.090	0.765	Two-way
	Diet	1	4747	4747	0.969	0.326	ANOVA
	Water × Diet	1	271	271	0.055	0.814	(rank trans-
	Residuals	237	1160942	4898			formed)
SAFA content	*Water*	1	50	49.67	0.357	0.554	Two-way
	Diet	1	1	1.32	0.009	0.923	ANOVA
	Water × Diet	1	16	15.61	0.112	0.740	(rank trans-
	Residuals	35	4873	139.24			formed)
MUFA content	*Water*	1	52.9	52.85	1.529	0.224	Two-way
	Diet	1	5.8	5.79	0.168	0.685	ANOVA
	Water × Diet	1	32.6	32.59	0.943	0.338	
	Residuals	35	1209.5	34.56			
PUFA content	*Water*	1	45	45.26	0.335	0.566	Two-way
	Diet	1	160	159.82	1.184	0.284	ANOVA
	Water × Diet	1	9	9.30	0.069	0.794	(rank trans-
	Residuals	35	4726	135.02			formed)
NLFA compo-	*Water*	1	0.003972	0.01335	0.491	0.736	PERMANOVA
sition of	*Diet*	1	0.005252	0.01766	0.649	0.604	(square root
gammarids	*Water × Diet*	1	0.004957	0.01667	0.613	0.615	transformed)
	Residuals	35	0.283244	0.95232			

Conclusion

The present study shows that AZO is not only moderately to highly toxic to amphipod shredders (cf. Zubrod et al., 2019), but probably also functions as metabolic disruptor – a yet overlooked mode of action in invertebrates. Therefore, further studies should target the underlying physiological mechanisms of the lipid, protein and glycogen catabolism. Moreover, it is evident from the present study that the relevance of waterborne and dietary exposure depends strongly on the concentrations applied as well as the sensitivity of the leaf-associated microbial community. The dietary pathway appears to be more relevant at lower AZO concentrations. The latter may be relevant in the field, as fungicide concentrations in the lower µg/L range have frequently been reported and can be detected even during the base flow in agricultural streams (e.g., Rabiet et al., 2010). In contrast, the higher test concentration can be considered as worst case (Berenzen et al., 2005). In the light of invasive fungal pathogens and global climate change (Stokstad, 2004; Elad and Pertot, 2014), which are expected to increase fungicide use, the exposure to fungicides and consequent waterborne and dietary effects are deemed increasingly relevant.

Acknowledgments

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Supporting information of Appendix A2

Table S1 Nominal and measured (means with 95 % CIs) AZO concentrations for the respective bioassays with the respective lowest calibration level (LCL).

Concen-tration (µg/L)	Test medium	Treatment	LCL (µg/L)	Fresh medium (µg/L)	3-day-old medium (µg/L)
30	Conditioning	Control	0.50	<LCL	-
		AZO		36.06 (30.39 to 41.72)	-
	SAM-5S	Control	0.50	<LCL	-
		Water		34.74 (28.14 to 41.34)	31.88 (26.62 to 37.15)
		Diet		-	0.90 (0.73 to 1.07)
		Combined		See Water	31.26 (25.66 to 36.86)
15	Conditioning	Control	1.00	<LCL	-
		AZO		15.02 (13.43 to 16.61)	-
	SAM-5S	Control	1.00	<LCL	-
		Water		16.49 (13.92 to 19.07)	12.43 (10.71 to 14.15)
		Diet		-	1.43 (1.17 to 1.69)
		Combined		See Water	14.64 (14.20 to 15.08)

Table S2 Results of logistic regression survival and molting analyses for *G. fossarum* subjected to different effect pathways during the 24-day bioassays with 30 and 15 µg AZO/L. All *p*-values <0.05 are printed in bold.

Concen-tration (µg/L)	Endpoint	Factorial predictor	Est.	SE	z-value	*p*-value	odds ratio
30	Survival	(Intercept)	-3.135	0.722	-4.342	<**0.001**	
		Water	2.968	0.778	3.815	<**0.001**	19.462
		Diet	<0.001	1.021	0.000	1.000	1.000
		Water × *Diet*	0.418	1.101	0.380	0.704	1.519
	Molting	(Intercept)	0.087	0.295	0.295	0.768	
		Water	1.618	0.619	2.615	**0.009**	5.042
		Diet	0.447	0.425	1.053	0.293	1.564
		Water × *Diet*	0.844	1.235	0.683	0.494	2.325
15	Survival	(Intercept)	-3.029	0.591	-5.123	<**0.001**	
		Water	-0.422	0.930	-0.453	0.650	0.656
		Diet	0.304	0.785	0.387	0.699	1.355
		Water × *Diet*	-0.304	1.284	-0.237	0.813	0.738
	Molting	(Intercept)	1.232	0.304	4.056	<**0.001**	
		Water	-0.467	0.407	-1.147	0.251	0.627
		Diet	-0.588	0.406	-1.448	0.148	0.556
		Water × *Diet*	0.739	0.562	1.314	0.189	2.093

Est. (Estimate also known as *b* coefficient); SE (standard error); z-value (outcome of z-statistic); odd ratio (indicates the influence of the factorial predictor on gammarids' mortality)

Table S3 Estimates of Kaplan-Meier survival analysis for *G. fossarum* subjected to different effect pathways during the 24-day bioassays with 30 and 15 µg AZO/L.

Concen-tration (µg/L)	Treatment	Day *i*	Survivors at day *i*	Deaths at day *i*	Survival proba-bility	±95 % CI
30	Control	21	48	1	0.98	0.94 to 1.00
		24	47	1	0.96	0.90 to 1.00
	Water	3	48	1	0.98	0.94 to 1.00
		6	47	2	0.94	0.87 to 1.00
		9	45	6	0.81	0.71 to 0.93
		12	39	3	0.75	0.64 to 0.88
		15	36	3	0.69	0.57 to 0.83
		18	33	3	0.63	0.50 to 0.78
		21	30	3	0.56	0.44 to 0.72
		24	27	1	0.54	0.42 to 0.70
	Diet	9	48	1	0.98	0.94 to 1.00
		15	47	1	0.96	0.90 to 1.00
	Combined	3	48	1	0.98	0.94 to 1.00
		6	47	2	0.94	0.87 to 1.00
		9	45	3	0.88	0.79 to 0.97
		12	42	2	0.83	0.73 to 0.95
		15	40	9	0.65	0.52 to 0.80
		18	31	4	0.56	0.44 to 0.72
		21	27	3	0.50	0.38 to 0.66
		24	24	3	0.44	0.32 to 0.60
15	Control	21	65	1	0.99	0.96 to 1.00
		24	64	2	0.95	0.90 to 1.00
	Water	6	65	1	0.99	0.96 to 1.00
		12	64	1	0.97	0.93 to 1.00
	Diet	15	65	1	0.99	0.96 to 1.00
		18	64	2	0.95	0.90 to 1.00
		21	62	1	0.94	0.88 to 1.00
	Combined	6	65	1	0.99	0.96 to 1.00
		24	64	1	0.97	0.93 to 1.00

Table S4 ANOVA-tables for gammarids' neutral lipid fatty acid (NLFA) contents during the 24-day bioassays with 30 (n = 6) and 15 (n = 10) µg AZO/L.

Concen-tration (µg/L)	Fatty acid	Factorial predictor	df1	SS	MS	*F*-value	*p*-value
30	12:0	*Water*	1	34.15	34.152	0.694	0.415
		Diet	1	13.02	13.023	0.265	0.613
		Water × *Diet*	1	29.79	29.792	0.605	0.446
		Residuals	19	935.03	49.212		
	13:0	*Water*	1	0.00	0.000	0.000	1.000
		Diet	1	6.29	6.286	0.120	0.733
		Water × *Diet*	1	12.01	12.014	0.230	0.637
		Residuals	19	993.7	52.3		
	14:0	*Water*	1	0.2968	0.29684	0.237	0.632
		Diet	1	0.1025	0.10252	0.082	0.778
		Water × *Diet*	1	0.892	1.25166	0.713	0.409
		Residuals	19	23.7816	1.252		
	15:0	*Water*	1	0.01881	0.018813	0.694	0.415
		Diet	1	0.00004	0.000035	0.001	0.972
		Water × *Diet*	1	0.03544	0.035443	1.307	0.267
		Residuals	19	0.51507	0.027109		
	16:0	*Water*	1	4.783	4.7835	0.436	0.517
		Diet	1	1.139	1.1389	0.104	0.751
		Water × *Diet*	1	5.285	5.2855	0.482	0.496
		Residuals	19	208.461	10.9716		
	17:0	*Water*	1	0.000642	0.0006421	0.039	0.846
		Diet	1	0.011137	0.0111367	0.676	0.421
		Water × *Diet*	1	0.00469	0.0046898	0.285	0.600
		Residuals	19	0.313245	0.0164866		
	18:0	*Water*	1	0.0465	0.046541	0.212	0.650
		Diet	1	0.0451	0.045117	0.206	0.655
		Water × *Diet*	1	0.0356	0.035592	0.162	0.692
		Residuals	19	4.1655	0.219239		
	20:0	*Water*	1	0.000192	0.000192	0.261	0.615
		Diet	1	0.0003751	0.0003751	0.510	0.484
		Water × *Diet*	1	0.0000737	7.371E-05	0.100	0.755
		Residuals	19	0.0139678	0.0007352		
	22:0	*Water*	1	0.0002093	0.0002093	0.408	0.530
		Diet	1	0.0003274	0.0003274	0.639	0.434
		Water × *Diet*	1	0.0000008	7.8E-07	0.002	0.969
		Residuals	19	0.0097352	0.0005124		
	14:1ω5	*Water*	1	1.57	1.568	0.030	0.864
		Diet	1	8.89	8.892	0.171	0.684
		Water × *Diet*	1	15.87	15.873	0.306	0.587
		Residuals	19	985.67	51.877		

Table S4 continued.

Concen-tration (µg/L)	Fatty acid	Factorial predictor	df1	SS	MS	*F*-value	*p*-value
30	16:1ω7	*Water*	1	0.2973	0.29729	0.418	0.526
		Diet	1	0.0691	0.06909	0.097	0.759
		Water × *Diet*	1	0.2496	0.24963	0.351	0.561
		Residuals	19	13.514	0.71126		
	18:1ω7	*Water*	1	0.034	0.034024	0.142	0.710
		Diet	1	0.0146	0.014603	0.061	0.808
		Water × *Diet*	1	0.0757	0.075695	0.316	0.581
		Residuals	19	4.5494	0.239442		
	18:1ω9	*Water*	1	10.76	10.762	0.208	0.654
		Diet	1	0.58	0.578	0.011	0.917
		Water × *Diet*	1	38.22	38.218	0.737	0.401
		Residuals	19	985.55	51.871		
	20:1ω9	*Water*	1	0.00044	0.0004377	0.019	0.891
		Diet	1	0.00803	0.0080252	0.355	0.559
		Water × *Diet*	1	0.01161	0.011614	0.513	0.483
		Residuals	19	0.43014	0.0226389		
	22:1ω9	*Water*	1	0.0008126	0.0008126	1.802	0.195
		Diet	1	0.0003059	0.0003059	0.678	0.420
		Water × *Diet*	1	0.000541	0.000541	1.199	0.287
		Residuals	19	0.0085705	0.0004511		
	18:2ω6	*Water*	1	0.368	0.3684	0.091	0.766
		Diet	1	0.204	0.2039	0.051	0.825
		Water × *Diet*	1	2.478	2.4781	0.613	0.443
		Residuals	19	76.753	4.0396		
	18:3ω3	*Water*	1	14.11	14.114	0.292	0.595
		Diet	1	36.25	36.251	0.751	0.397
		Water × *Diet*	1	44.27	44.268	0.917	0.350
		Residuals	19	917.37	48.282		
	18:3ω6	*Water*	1	0.0002762	0.0002762	0.355	0.559
		Diet	1	0.0001223	0.0001223	0.157	0.696
		Water × *Diet*	1	0.0003352	0.0003352	0.431	0.520
		Residuals	19	0.0147953	0.0007787		
	20:2ω6	*Water*	1	0.00125	0.0012497	0.070	0.794
		Diet	1	0.00000	0.0000002	0.000	0.997
		Water × *Diet*	1	0.01939	0.0193877	1.088	0.310
		Residuals	19	0.33873	0.0178278		
	20:3ω3	*Water*	1	14.11	14.114	0.288	0.598
		Diet	1	65.78	65.775	1.343	0.261
		Water × *Diet*	1	1.24	1.244	0.025	0.875
		Residuals	19	930.87	48.993		
	20:4ω6	*Water*	1	0.002533	0.0025332	0.330	0.572
		Diet	1	0.000416	0.000416	0.054	0.818
		Water × *Diet*	1	0.000031	0.0000313	0.004	0.950
		Residuals	19	0.145707	0.0076688		

Table S4 continued.

Concen-tration (µg/L)	Fatty acid	Factorial predictor	df1	SS	MS	*F*-value	*p*-value
30	20:5ω3	*Water*	1	0.00312	0.003121	0.082	0.778
		Diet	1	0.00003	0.000031	0.001	0.978
		Water × *Diet*	1	0.02147	0.021473	0.561	0.463
		Residuals	19	0.72727	0.038278		
	22:6ω3	*Water*	1	0.0009977	0.00099773	0.724	0.405
		Diet	1	0.0023741	0.00237407	1.724	0.205
		Water × *Diet*	1	0.0008197	0.00081972	0.595	0.450
		Residuals	19	0.0261684	0.00137729		
15	12:0	*Water*	1	10	10.26	0.074	0.787
		Diet	1	82	82.04	0.592	0.447
		Water × *Diet*	1	1	0.94	0.007	0.935
		Residuals	35	4847	138.48		
	13:0	*Water*	1	0.000137	0.0001366	0.409	0.527
		Diet	1	0.000009	0.0000091	0.027	0.870
		Water × *Diet*	1	0.000027	0.0000272	0.082	0.777
		Residuals	35	0.011687	0.0003339		
	14:0	*Water*	1	75	74.82	0.548	0.464
		Diet	1	40	39.9	0.292	0.592
		Water × *Diet*	1	46	46.38	0.340	0.564
		Residuals	35	4779	136.54		
	15:0	*Water*	1	0.0035	0.003539	0.345	0.561
		Diet	1	0.0029	0.002903	0.283	0.598
		Water × *Diet*	1	0.003	0.002955	0.288	0.595
		Residuals	35	0.3587	0.010249		
	16:0	*Water*	1	64	64.14	0.463	0.501
		Diet	1	13	13.21	0.095	0.759
		Water × *Diet*	1	16	16.44	0.119	0.732
		Residuals	35	4846	138.46		
	17:0	*Water*	1	33	33.25	0.245	0.623
		Diet	1	52	51.87	0.383	0.540
		Water × *Diet*	1	111	111.38	0.822	0.371
		Residuals	35	4744	135.53		
	18:0	*Water*	1	112	111.77	0.845	0.364
		Diet	1	118	117.81	0.891	0.352
		Water × *Diet*	1	82	81.83	0.619	0.437
		Residuals	35	4629	132.25		
	20:0	*Water*	1	54	54.29	0.390	0.537
		Diet	1	0	0.26	0.002	0.965
		Water × *Diet*	1	9	8.99	0.065	0.801
		Residuals	35	4876	139.33		
	21:0	*Water*	1	43	43.13	0.324	0.573
		Diet	1	222	222.45	1.672	0.204
		Water × *Diet*	1	18	18.17	0.137	0.714
		Residuals	35	4656	133.02		

Table S4 continued.

Concen-tration (µg/L)	Fatty acid	Factorial predictor	df1	SS	MS	*F*-value	*p*-value
15	22:0	*Water*	1	0	0.23	0.002	0.968
		Diet	1	57	56.53	0.408	0.527
		Water × *Diet*	1	35	34.82	0.251	0.619
		Residuals	35	4848	138.51		
	23:0	*Water*	1	0	0.23	0.002	0.967
		Diet	1	83	83.19	0.609	0.440
		Water × *Diet*	1	76	76.43	0.560	0.459
		Residuals	35	4780	136.56		
	24:0	*Water*	1	64	64.14	0.482	0.492
		Diet	1	120	119.83	0.900	0.349
		Water × *Diet*	1	12	12.25	0.092	0.763
		Residuals	35	4661	133.18		
	14:1ω5	*Water*	1	0.00233	0.0023332	2.454	0.126
		Diet	1	0.00001	0.0000059	0.006	0.938
		Water × *Diet*	1	0.00009	0.0000884	0.093	0.762
		Residuals	35	0.03327	0.0009506		
	16:1ω7	*Water*	1	105	105.09	0.764	0.388
		Diet	1	9	8.61	0.063	0.804
		Water × *Diet*	1	13	13.37	0.097	0.757
		Residuals	35	4813	137.51		
	18:1ω7	*Water*	1	0.156	0.15648	0.935	0.340
		Diet	1	0.044	0.04404	0.263	0.611
		Water × *Diet*	1	0.261	0.26124	1.561	0.220
		Residuals	35	5.856	0.16731		
	18:1ω9	*Water*	1	29.7	29.687	1.350	0.253
		Diet	1	5.1	5.084	0.231	0.634
		Water × *Diet*	1	21.4	21.388	0.973	0.331
		Residuals	35	769.4	21.984		
	20:1ω9	*Water*	1	0.0019	0.00191	0.083	0.775
		Diet	1	0.0151	0.01511	0.656	0.423
		Water × *Diet*	1	0.0566	0.0566	2.457	0.126
		Residuals	35	0.8062	0.02303		
	22:1ω9	*Water*	1	0.00067	0.00067	0.355	0.555
		Diet	1	0.00392	0.003919	2.077	0.158
		Water × *Diet*	1	0.00106	0.001063	0.563	0.458
		Residuals	35	0.06605	0.001887		
	18:2ω6	*Water*	1	2.09	2.0933	1.112	0.299
		Diet	1	0.01	0.0124	0.007	0.936
		Water × *Diet*	1	1.11	1.1132	0.591	0.447
		Residuals	35	65.88	1.8822		
	18:3ω3	*Water*	1	2.01	2.007	0.462	0.501
		Diet	1	11.93	11.933	2.750	0.106
		Water × *Diet*	1	0.85	0.846	0.195	0.662
		Residuals	35	151.9	4.34		

Table S4 continued.

Concen-tration (µg/L)	Fatty acid	Factorial predictor	df1	SS	MS	*F*-value	*p*-value
15	18:3ω6	*Water*	1	0.001072	0.0010724	2.246	0.143
		Diet	1	0.003103	0.0031025	6.499	0.015
		Water × *Diet*	1	0.000053	0.0000533	0.112	0.740
		Residuals	35	0.016709	0.0004774		
	20:2ω6	*Water*	1	0.0001	0.000069	0.007	0.935
		Diet	1	0.0017	0.001707	0.170	0.682
		Water × *Diet*	1	0.0182	0.018184	1.815	0.187
		Residuals	35	0.3507	0.01002		
	20:3ω3	*Water*	1	0	0.41	0.003	0.956
		Diet	1	278	278.27	2.098	0.156
		Water × *Diet*	1	19	18.62	0.140	0.710
		Residuals	35	4643	132.65		
	20:3ω6	*Water*	1	9	9.26	0.068	0.795
		Diet	1	10	9.76	0.072	0.790
		Water × *Diet*	1	152	152.24	1.121	0.297
		Residuals	35	4751	135.75		
	20:4ω6	*Water*	1	0.0004	0.000386	0.029	0.867
		Diet	1	0.0010	0.001039	0.077	0.783
		Water × *Diet*	1	0.0273	0.027298	2.022	0.164
		Residuals	35	0.4725	0.0135		
	20:5ω3	*Water*	1	5	5.03	0.037	0.849
		Diet	1	1	1.5	0.011	0.917
		Water × *Diet*	1	157	156.98	1.150	0.291
		Residuals	35	4776	136.47		
	22:2ω6	*Water*	1	246	246.4	1.967	0.170
		Diet	1	187	187.3	1.495	0.230
		Water × *Diet*	1	111	111	0.886	0.353
		Residuals	35	4385	125.3		
	22:6ω3	*Water*	1	86	86.31	0.659	0.422
		Diet	1	254	254.33	1.942	0.172
		Water × *Diet*	1	15	15.07	0.115	0.737
		Residuals	35	4584	130.98		

Table S5 Group medians (with 95 % CIs) of neutral lipid fatty acids (NLFAs) of *G. fossarum* (µg/mg gammarid dry mass) during the 24-day bioassays with 30 (n = 6) and 15 (n = 10) µg AZO/L.

Concen-tration (µg/L	Fatty acid(s)	Treatment	Median	±95 % CI
30	12:0	Control	0.36	0.24 to 0.81
		Water	0.44	0.19 to 0.56
		Diet	0.25	0.20 to 0.53
		Combined	0.39	0.23 to 0.92
	13:0	Control	0.05	0.04 to 0.09
		Water	0.05	0.03 to 0.07
		Diet	0.04	0.02 to 0.08
		Combined	0.05	0.04 to 0.10
	14:0	Control	2.00	1.24 to 4.62
		Water	2.64	0.70 to 3.03
		Diet	1.52	0.80 to 2.80
		Combined	2.08	1.10 to 4.87
	15:0	Control	0.43	0.37 to 0.84
		Water	0.50	0.26 to 0.66
		Diet	0.41	0.16 to 0.61
		Combined	0.54	0.37 to 0.73
	16:0	Control	7.40	6.05 to 16.05
		Water	9.66	4.28 to 12.5
		Diet	6.88	3.54 to 11.16
		Combined	8.38	6.10 to 14.74
	17:0	Control	0.36	0.29 to 0.65
		Water	0.47	0.22 to 0.49
		Diet	0.34	0.15 to 0.52
		Combined	0.37	0.24 to 0.58
	18:0	Control	1.11	0.55 to 1.96
		Water	1.27	0.52 to 1.56
		Diet	0.78	0.44 to 1.50
		Combined	1.06	0.58 to 1.82
	20:0	Control	0.06	0.05 to 0.09
		Water	0.08	0.04 to 0.09
		Diet	0.04	0.03 to 0.13
		Combined	0.07	0.02 to 0.09
	22:0	Control	0.04	0.03 to 0.07
		Water	0.03	0.01 to 0.07
		Diet	0.03	0.02 to 0.08
		Combined	0.03	0.02 to 0.04
	SAFA	Control	11.62	9.42 to 25.17
	content	Water	15.28	6.44 to 18.50
		Diet	10.39	5.38 to 17.28
		Combined	13.03	8.71 to 23.90

Table S5 continued.

Concen-tration (µg/L	Fatty acid(s)	Treatment	Median	±95 % CI
30	14:1ω5	Control	0.05	0.03 to 0.10
		Water	0.05	0.02 to 0.08
		Diet	0.04	0.02 to 0.15
		Combined	0.06	0.03 to 0.13
	16:1ω7	Control	1.84	1.67 to 3.21
		Water	2.37	0.98 to 3.17
		Diet	1.85	0.82 to 3.50
		Combined	2.01	1.47 to 3.91
	18:1ω7	Control	1.36	1.20 to 2.30
		Water	1.63	0.73 to 2.15
		Diet	1.40	0.68 to 2.32
		Combined	1.59	1.20 to 2.30
	18:1ω9	Control	15.33	10.93 to 26.95
		Water	15.92	3.71 to 29.76
		Diet	11.94	7.62 to 23.04
		Combined	17.66	11.36 to 30.04
	20:1ω9	Control	0.29	0.16 to 0.49
		Water	0.32	0.06 to 0.44
		Diet	0.31	0.13 to 0.48
		Combined	0.27	0.21 to 0.68
	22:1ω9	Control	0.04	0.04 to 0.06
		Water	0.04	0.03 to 0.09
		Diet	0.04	0.02 to 0.06
		Combined	0.05	0.03 to 0.09
	MUFA	Control	18.86	14.04 to 33.02
	content	Water	20.38	5.54 to 35.68
		Diet	14.85	9.29 to 29.55
		Combined	21.99	14.32 to 37.15
	18:2ω6	Control	4.92	3.72 to 8.01
		Water	5.45	1.16 to 7.38
		Diet	4.07	1.84 to 6.93
		Combined	5.05	3.46 to 8.43
	18:3ω3	Control	3.17	2.84 to 6.90
		Water	3.89	2.87 to 6.99
		Diet	4.14	1.77 to 5.30
		Combined	3.22	2.70 to 6.16
	18:3ω6	Control	0.05	0.03 to 0.08
		Water	0.06	0.01 to 0.08
		Diet	0.06	0.00 to 0.09
		Combined	0.05	0.03 to 0.11
	20:2ω6	Control	0.36	0.24 to 0.48
		Water	0.37	0.05 to 0.48
		Diet	0.25	0.14 to 0.48
		Combined	0.33	0.23 to 0.54

Table S5 continued.

Concen-tration (µg/L	Fatty acid(s)	Treatment	Median	±95 % CI
30	20:3ω3	Control	0.21	0.15 to 0.34
		Water	0.24	0.14 to 0.57
		Diet	0.18	0.08 to 0.38
		Combined	0.18	0.15 to 0.39
	20:4ω6	Control	0.13	0.08 to 0.22
		Water	0.16	0.02 to 0.37
		Diet	0.13	0.04 to 0.24
		Combined	0.13	0.06 to 0.29
	20:5ω3	Control	0.32	0.19 to 0.45
		Water	0.40	0.11 to 0.72
		Diet	0.34	0.09 to 0.75
		Combined	0.30	0.18 to 0.57
	22:6ω3	Control	0.04	0.01 to 0.08
		Water	0.05	0.02 to 0.17
		Diet	0.03	0.02 to 0.07
		Combined	0.02	0.00 to 0.09
	PUFA	Control	8.86	7.82 to 16.52
	content	Water	12.15	5.61 to 13.83
		Diet	9.23	3.99 to 14.23
		Combined	9.12	7.08 to 16.50
15	12:0	Control	0.44	0.36 to 0.71
		Water	0.48	0.42 to 0.62
		Diet	0.52	0.22 to 1.03
		Combined	0.52	0.44 to 0.86
	13:0	Control	0.05	0.03 to 0.05
		Water	0.05	0.03 to 0.05
		Diet	0.05	0.03 to 0.07
		Combined	0.04	0.03 to 0.06
	14:0	Control	2.33	1.56 to 3.11
		Water	2.76	1.73 to 3.42
		Diet	2.89	1.14 to 5.70
		Combined	2.72	2.21 to 3.26
	15:0	Control	0.38	0.34 to 0.45
		Water	0.39	0.33 to 0.48
		Diet	0.38	0.27 to 0.53
		Combined	0.33	0.28 to 0.48
	16:0	Control	9.08	8.2 to 10.46
		Water	9.25	6.9 to 10.47
		Diet	9.64	5.97 to 17.43
		Combined	9.42	6.86 to 10.32
	17:0	Control	0.37	0.29 to 0.66
		Water	0.43	0.30 to 0.53
		Diet	0.44	0.26 to 0.55
		Combined	0.34	0.23 to 0.55

Table S5 continued.

Concen-tration (µg/L	Fatty acid(s)	Treatment	Median	±95 % CI
15	18:0	Control	1.01	0.54 to 1.76
		Water	0.96	0.76 to 1.31
		Diet	0.93	0.67 to 1.46
		Combined	0.76	0.55 to 1.01
	20:0	Control	0.09	0.05 to 0.14
		Water	0.08	0.03 to 0.15
		Diet	0.11	0.02 to 0.17
		Combined	0.09	0.02 to 0.14
	21:0	Control	0.03	0.01 to 0.14
		Water	0.02	0.01 to 0.04
		Diet	0.02	0.00 to 0.05
		Combined	0.02	0.00 to 0.08
	22:0	Control	0.04	0.02 to 0.44
		Water	0.06	0.03 to 0.23
		Diet	0.05	0.02 to 0.14
		Combined	0.05	0.01 to 0.15
	23:0	Control	0.04	0.01 to 0.33
		Water	0.05	0.02 to 0.12
		Diet	0.04	0.01 to 0.11
		Combined	0.04	0.01 to 0.06
	24:0	Control	0.05	0.00 to 1.54
		Water	0.06	0.00 to 0.39
		Diet	0.03	0.00 to 0.29
		Combined	0.01	0.00 to 0.19
	SAFA	Control	14.98	12.04 to 16.01
	content	Water	14.98	10.72 to 16.98
		Diet	14.95	8.99 to 27.07
		Combined	14.58	10.24 to 16.01
	14:1ω5	Control	0.06	0.03 to 0.11
		Water	0.05	0.03 to 0.06
		Diet	0.05	0.02 to 0.13
		Combined	0.05	0.03 to 0.07
	16:1ω7	Control	2.30	2.01 to 3.86
		Water	2.19	1.86 to 2.58
		Diet	2.53	1.82 to 4.34
		Combined	2.33	1.70 to 2.52
	18:1ω7	Control	1.53	1.28 to 1.98
		Water	1.64	1.46 to 2.00
		Diet	1.72	1.23 to 2.54
		Combined	1.45	1.01 to 1.77
	18:1ω9	Control	15.31	12.44 to 17.83
		Water	15.40	11.49 to 18.70
		Diet	15.57	12.53 to 28.86
		Combined	15.38	10.52 to 16.72

Table S5 continued.

Concen-tration (µg/L	Fatty acid(s)	Treatment	Median	±95 % CI
15	20:1ω9	Control	0.39	0.24 to 0.44
		Water	0.45	0.26 to 0.52
		Diet	0.43	0.29 to 0.68
		Combined	0.43	0.26 to 0.50
	22:1ω9	Control	0.07	0.01 to 0.14
		Water	0.02	0.00 to 0.11
		Diet	0.04	0.00 to 0.07
		Combined	0.03	0.00 to 0.08
	MUFA	Control	19.72	17.03 to 23.67
	content	Water	19.72	15.44 to 24.34
		Diet	20.24	16.08 to 36.70
		Combined	19.83	13.66 to 21.24
	18:2ω6	Control	4.19	3.21 to 4.96
		Water	4.03	3.34 to 5.23
		Diet	4.07	3.19 to 6.75
		Combined	3.73	2.30 to 5.12
	18:3ω3	Control	6.03	3.76 to 6.76
		Water	5.57	4.51 to 6.10
		Diet	6.80	4.52 to 8.84
		Combined	6.92	3.78 to 8.35
	18:3ω6	Control	0.02	0.00 to 0.05
		Water	0.01	0.00 to 0.02
		Diet	0.03	0.01 to 0.07
		Combined	0.02	0.00 to 0.06
	20:2ω6	Control	0.33	0.28 to 0.38
		Water	0.39	0.31 to 0.52
		Diet	0.37	0.26 to 0.54
		Combined	0.36	0.26 to 0.41
	20:3ω3	Control	0.40	0.25 to 0.51
		Water	0.43	0.36 to 0.49
		Diet	0.50	0.19 to 0.73
		Combined	0.51	0.32 to 0.66
	20:3ω6	Control	0.02	0.00 to 0.04
		Water	0.03	0.00 to 0.04
		Diet	0.02	0.00 to 0.05
		Combined	0.01	0.00 to 0.04
	20:4ω6	Control	0.30	0.16 to 0.41
		Water	0.34	0.27 to 0.45
		Diet	0.35	0.23 to 0.45
		Combined	0.31	0.16 to 0.43
	20:5ω3	Control	0.36	0.25 to 0.47
		Water	0.42	0.29 to 0.65
		Diet	0.40	0.26 to 0.68
		Combined	0.36	0.27 to 0.45

Table S5 continued.

Concen-tration (µg/L	Fatty acid(s)	Treatment	Median	±95 % CI
15	22:2ω6	Control	0.02	0.01 to 0.11
		Water	0.02	0.00 to 0.03
		Diet	0.01	0.00 to 0.03
		Combined	0.02	0.01 to 0.03
	22:6ω3	Control	0.08	0.04 to 0.11
		Water	0.06	0.03 to 0.09
		Diet	0.06	0.02 to 0.11
		Combined	0.05	0.04 to 0.07
	PUFA	Control	11.80	8.49 to 13.16
	content	Water	11.40	10.09 to 12.90
		Diet	12.90	9.02 to 17.74
		Combined	12.26	7.66 to 16.16

Saturated fatty acid (SAFA); Monounsaturated fatty acid (MUFA); Polyunsaturated fatty acid (PUFA)

Table S6 ANOVA-tables for gammarids' amino acid (AA; n = 6) contents during the 24-day bioassays with 30 µg AZO/L. All p-values <0.05 are printed in bold.

Amino acid	Factorial predictor	df1	SS	MS	F-value	p-value
Alanine	*Water*	1	55.02	55.02	8.213	**0.010**
	Diet	1	0.20	0.20	0.029	0.866
	Water × *Diet*	1	11.22	11.22	1.674	0.210
	Residuals	20	133.98	6.7		
Asparagine/	*Water*	1	150.5	150.50	5.484	**0.030**
Methionine	*Diet*	1	7.5	7.53	0.275	0.606
	Water × *Diet*	1	20.6	20.56	0.749	0.397
	Residuals	20	548.9	27.44		
Glutamic acid/	*Water*	1	394.5	394.5	7.483	**0.013**
Phenylalanine	*Diet*	1	28.8	28.8	0.547	0.468
	Water × *Diet*	1	118.2	118.2	2.243	0.150
	Residuals	20	1054.5	52.7		
Glycine	*Water*	1	27.36	27.357	5.095	**0.035**
	Diet	1	0.45	0.449	0.084	0.776
	Water × *Diet*	1	26.02	26.023	4.846	**0.040**
	Residuals	20	107.39	5.369		
Histidine	*Water*	1	65.35	65.35	4.059	**0.001**
	Diet	1	3.42	3.42	0.735	0.401
	Water × *Diet*	1	20.89	20.89	4.493	**0.047**
	Residuals	20	92.97	4.65		

Table S6 continued.

Amino acid	Factorial predictor	df1	SS	MS	*F*-value	*p*-value
Isoleucine	*Water*	1	40.22	40.22	7.987	**0.010**
	Diet	1	0.16	0.16	0.032	0.859
	Water × *Diet*	1	15.30	15.30	3.039	0.097
	Residuals	20	100.70	5.03		
Leucine	*Water*	1	122.70	122.70	8.891	**0.007**
	Diet	1	0.19	0.19	0.014	0.907
	Water × *Diet*	1	55.29	55.29	4.006	0.059
	Residuals	20	276.00	13.80		
Lysine	*Water*	1	149.80	149.80	5.146	**0.035**
	Diet	1	3.20	3.16	0.109	0.745
	Water × *Diet*	1	23.70	23.70	0.814	0.378
	Residuals	20	582.20	29.11		
Proline	*Water*	1	37.00	37.00	9.547	**0.006**
	Diet	1	0.31	0.31	0.079	0.782
	Water × *Diet*	1	25.66	25.66	6.619	0.018
	Residuals	20	77.52	3.88		
Serine	*Water*	1	30.64	30.644	5.282	**0.033**
	Diet	1	0.01	0.012	0.002	0.964
	Water × *Diet*	1	0.26	0.258	0.044	0.835
	Residuals	20	116.04	5.802		
Threonine	*Water*	1	28.71	28.711	7.551	**0.012**
	Diet	1	0.73	0.730	0.192	0.666
	Water × *Diet*	1	7.77	7.771	2.044	0.168
	Residuals	20	76.05	3.803		
Tyrosine	*Water*	1	37.10	37.10	2.665	**0.002**
	Diet	1	0.41	0.41	0.141	0.712
	Water × *Diet*	1	10.73	10.73	3.662	0.070
	Residuals	20	58.58	2.93		
Valine	*Water*	1	55.31	55.31	7.605	**0.012**
	Diet	1	5.69	5.69	0.783	0.387
	Water × *Diet*	1	45.61	45.61	6.272	**0.021**
	Residuals	20	145.46	7.27		

Table S7 Group medians (with 95 % CIs) of amino acids (AAs; *n* = 6) of *G. fossarum* (µg/mg gammarid dry mass) during the 24-day bioassay with 30 µg AZO/L.

Amino acid(s)	Treatment	Median	±95 % CI
Alanine	Control	21.05	16.61 to 23.42
	Water	23.73	21.75 to 29.75
	Diet	21.42	18.93 to 24.63
	Combined	22.84	20.52 to 27.05
Asparagine/	Control	35.95	28.62 to 42.13
Methionine	Water	42.07	34.17 to 50.11
	Diet	35.98	31.44 to 42.74
	Combined	39.70	32.08 to 45.83
Glutamic acid/	Control	49.40	40.42 to 58.18
Phenylalanine	Water	63.41	49.29 to 71.97
	Diet	50.14	44.98 to 59.57
	Combined	55.12	46.79 to 64.34
Glycine	Control	17.61	13.23 to 19.18
	Water	20.78	17.04 to 24.76
	Diet	18.37	17.22 to 19.77
	Combined	18.36	16.22 to 21.54
Histidine	Control	10.55	9.60 to 13.47
	Water	15.43	12.12 to 22.09
	Diet	11.99	10.41 to 13.22
	Combined	13.51	11.11 to 16.46
Isoleucine	Control	15.62	12.51 to 17.35
	Water	19.92	14.65 to 22.21
	Diet	16.09	13.92 to 18.99
	Combined	17.76	14.46 to 19.92
Leucine	Control	26.13	21.06 to 29.61
	Water	34.06	25.28 to 38.17
	Diet	27.98	25.42 to 32.17
	Combined	30.48	25.85 to 34.29
Lysine	Control	27.65	20.30 to 32.31
	Water	31.17	25.71 to 46.39
	Diet	28.76	21.59 to 39.33
	Combined	31.57	26.91 to 37.14
Proline	Control	14.00	11.17 to 15.30
	Water	19.03	14.15 to 20.94
	Diet	14.83	13.67 to 17.41
	Combined	15.88	14.12 to 16.87
Serine	Control	15.97	12.99 to 18.27
	Water	17.13	15.76 to 22.91
	Diet	15.55	11.19 to 20.44
	Combined	17.53	16.11 to 19.91

Table S7 continued.

Amino acid(s)	Treatment	Median	±95 % CI
Threonine	Control	14.58	11.76 to 16.37
	Water	17.72	14.03 to 20.42
	Diet	14.93	12.56 to 17.65
	Combined	15.83	13.37 to 17.59
Tyrosine	Control	9.46	7.36 to 11.04
	Water	13.62	9.76 to 15.40
	Diet	10.17	8.22 to 11.86
	Combined	11.50	9.54 to 12.95
Valine	Control	18.27	15.06 to 20.90
	Water	25.47	17.82 to 28.20
	Diet	22.07	19.61 to 23.19
	Combined	22.16	17.81 to 23.92
Total AA	Control	277.43	220.86 to 317.54
content	Water	349.99	275.52 to 402.62
	Diet	286.01	257.52 to 339.41
	Combined	313.29	273.16 to 354.88

Table S8 Number of analyzed replicates and group medians (with 95 % CIs) of parameters describing leaf quality used during the 24-day bioassays. Moreover, the statistical tests used for the respective endpoint as well as the *p*-value from the statistical comparison of 30 or 15 µg AZO/L with the control is shown. All *p*-values below 0.05 are printed in bold.

Endpoint	Concen-tration (µg/L)	*n*	Median	±95 % CI	Statistical test	*p*-value
Fungal biomass in µg ergosterol/g leaf dry mass	0	8	29.38	8.16 to 54.16	Student's *t*	
	30	8	38.78	11.58 to 89.41		0.128
Bacterial density in 10^9 cells/g leaf dry mass	0	8	0.67	0.24 to 2.44	Wilcoxon rank-sum	
	30	8	0.77	0.28 to 2.08		0.645
Fungal biomass in µg ergosterol/g leaf dry mass	0	12	61.04	42.49 to 107.72	Student's *t*	
	15	11	64.53	17.56 to 107.83		0.830
Bacterial density in 10^9 cells/g leaf dry mass	0	12	0.59	0.41 to 0.83	Wilcoxon rank-sum	
	15	12	0.71	0.38 to 0.92		0.799
Total FA content in µg/mg leaf dry mass	0	12	10.76	9.95 to 13.94	Wilcoxon rank-sum test	
	15	12	15.02	11.19 to 16.97		**0.033**
Saturated FA content in µg/mg leaf dry mass	0	12	4.45	4.04 to 5.90	Wilcoxon rank-sum test	
	15	12	5.71	4.77 to 6.96		0.068
Monounsaturated FA content in µg/mg leaf dry mass	0	12	1.64	1.12 to 2.08	Student's *t*	
	15	12	2.12	1.72 to 2.36		**0.019**
Polyunsaturated FA content in µg/mg leaf dry mass	0	12	4.95	4.41 to 6.23	Wilcoxon rank-sum test	
	15	12	6.89	5.11 to 8.11		0.078
FA composition of leaves	0	12	-	-	PERMANOVA	
	15	12	-	-		**0.023**

Table S9 Group medians (with 95 % CIs) of total fatty acids (FAs) associated with leaves (µg/mg dry mass of leaf material) conditioned in the absence (n = 12) or presence of 15 µg AZO/L (n = 11) during 24-day bioassay. Moreover, the statistical test used for the respective FA as well as the p-value from the statistical comparison of 15 µg AZO/L with the control is shown. All p-values below 0.05 are printed in bold.

Fatty acid	Concen-tration (µg/L)	Median	±95 % CI	Test	p-value
12:0	0	0.01	0.00 to 0.01	Wilcoxon rank-sum test	
	15	0.01	0.00 to 0.02		0.143
14:0	0	0.31	0.27 to 0.42	Wilcoxon rank-sum test	
	15	0.40	0.35 to 0.53		**0.017**
15:0	0	0.04	0.04 to 0.06	Wilcoxon rank-sum test	
	15	0.05	0.05 to 0.06		0.178
16:0	0	2.06	1.87 to 2.55	Wilcoxon rank-sum test	
	15	2.85	2.28 to 3.47		0.078
18:0	0	0.61	0.52 to 1.07	Wilcoxon rank-sum test	
	15	0.77	0.62 to 0.94		0.219
20:0	0	0.53	0.43 to 0.60	Student's t	
	15	0.65	0.49 to 0.83		0.136
22:0	0	0.67	0.58 to 0.77	Wilcoxon rank-sum test	
	15	0.86	0.66 to 0.94		**0.033**
23:0	0	0.07	0.06 to 0.09	Wilcoxon rank-sum test	
	15	0.10	0.08 to 0.11		**0.005**
24:0	0	0.19	0.16 to 0.24	Wilcoxon rank-sum test	
	15	0.23	0.20 to 0.26		**0.045**
16:1ω7	0	0.74	0.42 to 0.94	Student's t	
	15	0.88	0.79 to 1.06		**0.029**
18:1ω7	0	0.50	0.30 to 0.65	Student's t	
	15	0.60	0.47 to 0.85		**0.046**
18:1ω9	0	0.36	0.32 to 0.45	Wilcoxon rank-sum test	
	15	0.43	0.39 to 0.47		0.052
20:1ω9	0	0.01	0.01 to 0.01	Wilcoxon rank-sum test	
	15	0.01	0.01 to 0.02		0.143
18:2ω6	0	0.84	0.75 to 0.94	Wilcoxon rank-sum test	
	15	1.17	0.84 to 1.46		**0.017**
18:3ω3	0	3.38	3.00 to 4.31	Wilcoxon rank-sum test	
	15	5.00	3.12 to 6.11		0.101
18:3ω6	0	0.02	0.02 to 0.02	Student's t	
	15	0.03	0.02 to 0.04		**0.006**
20:2ω6	0	0.02	0.01 to 0.02	Student's t	
	15	0.01	0.01 to 0.02		0.097
20:3ω3	0	0.03	0.02 to 0.06	Student's t	
	15	0.03	0.02 to 0.04		0.626
20:3ω6	0	0.02	0.01 to 0.02	Student's t	
	15	0.02	0.01 to 0.02		0.395

Table S9 continued.

Fatty acid	Concen-tration (µg/L)	Median	±95 % CI	Test	*p*-value
20:4ω6	0	0.14	0.09 to 0.24	Student's *t*	
	15	0.16	0.09 to 0.23		0.857
20:5ω3	0	0.19	0.14 to 0.33	Wilcoxon rank-sum test	
	15	0.21	0.14 to 0.26		0.932
22:2ω6	0	0.18	0.14 to 0.21	Student's *t*	
	15	0.15	0.14 to 0.19		0.503
22:6ω3	0	0.03	0.01 to 0.09	Wilcoxon rank-sum test	
	15	0.02	0.02 to 0.03		0.630

Appendix A3

Mixture effects of a fungicide and an antibiotic: assessment and prediction using a decomposer-detritivore system

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Abstract

Antimicrobials, such as fungicides and antibiotics, pose a risk for microbial decomposers (i.e., bacteria and aquatic fungi) and invertebrate detritivores (i.e., shredders) that play a pivotal role in the ecosystem function of leaf litter breakdown. Although waterborne toxicity and diet-related effects (i.e., dietary exposure and microorganism-mediated alterations in food quality for shredders) of fungicides and antibiotics on decomposer-detritivore systems have been increasingly documented, their joint effect is unknown. We therefore assessed waterborne and dietary effects of an antimicrobial mixture consisting of the fungicide azoxystrobin (AZO) and the antibiotic ciprofloxacin (CIP) on microbial decomposers and the shredder *Gammarus fossarum* using a tiered approach. We compared effect sizes measured in the present study with model predictions (i.e., independent action) based on published data. During a 7-day feeding activity assay quantifying waterborne toxicity in *G. fossarum*, the leaf consumption of gammarids was reduced by ~60 % compared to the control when subjected to the mixture at concentrations of each component causing a 20 % reduction in the same response variable when applied individually. Moreover, the selective feeding of gammarids during the food choice assay indicated alterations in food quality induced by the antimicrobial mixture. The food selection and, in addition, the decrease in microbial leaf decomposition is likely linked to changes in leaf-associated bacteria and fungi. During a long-term assay, energy processing, growth and energy reserves of gammarids were increased in presence of 15 and 500 µg/L of AZO and CIP, respectively, through the dietary pathway. These physiological responses were probably driven by CIP-induced alterations in the gut microbiome or immune system of gammarids. In general, model predictions matched observed effects caused by waterborne exposure on the leaf consumption, energy processing and growth of gammarids during short- and long-term assays, respectively. However, when complex horizontal (bacteria and aquatic fungi) and vertical (leaf-associated microorganisms and shredders) interactions were involved, model predictions partly over- or underestimated mixture effects. Therefore, the present study identifies uncertainties of mixture effect predictions for complex biological systems calling for studies targeting the underlying processes and mechanisms.

Keywords

Aquatic fungi; Azoxystrobin; Ciprofloxacin; *Gammarus*; Leaf litter breakdown

Introduction

The breakdown of leaf litter is an important process for the nutrient and energy cycling in stream ecosystems (Minshall, 1967; Fisher and Likens, 1973). Microbial decomposers (i.e., fungi and bacteria) and invertebrate detritivores (i.e., shredders) are fundamental for this ecosystem process (Gessner et al., 1999; Graça, 2001). Microbial decomposers contribute substantially to the mineralization of leaf litter (Hieber and Gessner, 2002). In particular aquatic fungi increase the nutritional quality and palatability of leaf litter for shredders (i.e., conditioning; Bärlocher and Kendrick, 1975; Graça et al., 1993). Shredders, in turn, transform leaf litter into fine particulate organic matter (e.g., feces) that are consumed by collectors (Bundschuh and McKie, 2016). Furthermore, the secondary production by shredders provides food for higher trophic levels (MacNeil et al., 1999).

These decomposers and detritivores as well as their interactions can be affected by chemical stressors (e.g., Fernández et al., 2015; Zubrod et al., 2017), among which antimicrobial substances (= antimicrobials), such as fungicides and antibiotics, are of particular interest for the following reasons: shredders can suffer from direct effects during waterborne exposure (e.g., Beketov and Liess, 2008; Bartlett et al., 2013) and dietary uptake of antimicrobials when adsorbed onto leaf litter (Zubrod et al., 2015c). Furthermore, due to their modes of action, which target vital processes in fungi (Ittner et al., 2018) and bacteria (Brandt et al., 2015), antimicrobials change the microbial decomposer community composition and consequently the palatability and nutritional quality of leaf litter for shredders (i.e., microorganism-mediated dietary effects; e.g., Hahn and Schulz, 2007; Zubrod et al., 2015c).

Even though fungicides and antibiotics can affect decomposer-detritivore systems, both chemical stressor groups have dissimilar effects on microbial decomposers. Fungicides, for instance, directly affect aquatic fungi (mainly aquatic hyphomycetes) and thereby reduce leaf litter quality for shredders (Zubrod et al., 2015c). Antibiotics, on the other hand, can release fungi from the competitive pressure by leaf-associated bacteria, increasing the growth of shredders indirectly (Bundschuh et al., 2017; Konschak et al., 2020). It is yet unknown how combined effects of these groups of antimicrobials affect decomposer-detritivore systems.

We address this issue by assessing effects of an antimicrobial mixture composed of a fungicide and an antibiotic using a well-established model decomposer-detritivore system. This system comprises a near-natural leaf-associated microbial decomposer community and

the amphipod shredder *Gammarus fossarum*. Using a tiered experimental approach, we first assessed the effects of short-term waterborne exposure on gammarids via a feeding activity assay. In a second step, we assessed for potential indirect effects and repellent effects (caused by adsorbed fungicides onto leaf litter; cf. Zubrod et al., 2015a) on shredders using a food choice assay and employing food selection as indicator of leaf palatability (Arsuffi and Suberkropp, 1989). Simultaneously, we determined effects on microbial decomposers by assessing their leaf decomposition activity. We further investigated long-term waterborne and diet-related effects of the antimicrobial mixture on the energy processing of gammarids (leaf consumption and feces production), growth and energy reserves using a full factorial design. Finally, we compared our observations to effect predictions of the independent action (IA) model (dealing with substances of dissimilar modes of action; Bliss, 1939) using data from previous publications (Zubrod et al., 2014, 2015a; Konschak et al., 2020, 2021). Thereby we assessed accuracy of effect predictions of antimicrobial mixtures with deviations (such as synergistic interactions) stimulating future research ultimately supporting a science based regulation of antimicrobial mixtures.

As IA models are designed to handle effects of substances with dissimilar modes of action in a given mixture, we expected its predictions to comply with the effects observed for *G. fossarum* when experiencing waterborne exposure. However, we hypothesized that effect predictions for leaf-associated microorganisms (i.e., decomposer community level) do not match the observed effects, as the IA model does not cover complex interactions between species belonging to the same or different trophic level(s) (i.e., horizontal and vertical interactions; e.g., Romaní et al., 2006).

Materials and methods

General overview

In a first step, waterborne effects on gammarids were determined following Zubrod et al. (2014): the assay was performed in May 2016 focusing on the feeding activity of *G. fossarum* exposed to the binary antimicrobial mixture comprised the fungicide azoxystrobin (AZO; mitochondrial respiratory chain inhibitor; Bartlett et al., 2002) and the antibiotic ciprofloxacin (CIP; DNA gyrase and topoisomerase IV inhibitor; Hooper and Wolfson, 1988; Fig 1a). CIP and AZO were selected in the previous studies by Konschak et al. (2020, 2021), as both antimicrobials are frequently detected in European surface waters (up to the µg/L range) and are toxic to highly toxic for aquatic organisms (Schreiner et al., 2016; Danner et al., 2019;

Zubrod et al., 2019). The concentrations selected for each mixture component (five AZO concentrations combined with a fixed CIP concentration) were below or similar to the concentration inducing a 20 % reduction (EC_{20}) in leaf consumption of gammarids when exposed individually (Table 1). The EC_{20} was selected as benchmark, as it is considered as an ecotoxicologically relevant concentration that provides an adequate protection for aquatic life (Barnthouse et al., 2008).

Table 1 Assays, source of experimental setups as well as nominal concentrations of the mixture components and the binary antimicrobial mixture for each assay.

Assay	Source of experimental setups	Mixture component	Nominal test concentration(s)	Mixture (AZO + CIP)	SI units
Feeding activity	Zubrod et al. (2014)	AZO	10.0; 27.5; 45.0; 62.5; 80.0	10.0 + 500; 27.5 + 500; 45.0 + 500; 62.5 + 500; 80.0 + 500	µg/L
	Konschak et al. (2020a)	CIP	500		µg/L
Food choice	Zubrod et al. (2015a)	AZO	0.1; 2.5	0.1 + 0.1; 0.1 + 2.5; 2.5 + 0.1;	mg/L
	Konschak et al. (2020a)	CIP	0.1; 2.5	2.5 + 2.5	mg/L
Long-term	Konschak et al. (2020b)	AZO	15.0	15.0 + 500.0	µg/L
	Konschak et al. (2020a)	CIP	500.0		µg/L

For the assessment of mixture effects on a leaf-associated microbial community, its microbial leaf decomposition and its indirect consequences on leaf palatability for shredders (Zubrod et al., 2015a), a food choice assay was performed in August 2017 (Fig. 1b). The antimicrobial mixture covered two concentrations of AZO and CIP in all possible combinations. These concentrations were set at 0.1 and 2.5 mg/L representing the lowest observed effect concentration for microbial decomposition and an overdosed concentration, respectively. The latter concentration should thus induce clear effects on the response variables (Zubrod et al., 2015a; Konschak et al., 2020).

In January 2018, a 24-day long-term assay was conducted to assess effects on the energy processing of gammarids, growth and energy reserves (i.e., neutral lipid fatty acids; NLFAs). The mixture effects via the waterborne and dietary pathway were assessed using a 2 × 2 factorial design (cf. Zubrod et al., 2015b): gammarids were not exposed via the water phase and fed with unexposed leaves (i.e., Control), exposed via the water phase and fed with unexposed leaves (i.e., Water), not exposed via the water phase and fed with exposed leaves (i.e., Diet), or subjected to both exposure pathways jointly (i.e., Combined; Fig. 1c). The

concentrations of the individual mixture components (i.e., 15 µg/L AZO and 500 µg/L CIP) selected for the present study resulted in sublethal effects in gammarids when tested in a similar set up individually (Table 1). Moreover, this choice allowed to test for compliance between predicted and observed effects.

Binary antimicrobial mixture

Individual stock solutions were prepared separately in the respective test media for AZO (Ortiva, Syngenta Agro GmbH, Basel, Switzerland; cf. Konschak et al., 2021) and CIP (98%, Acros Organics, Geel, Belgium; cf. Konschak et al., 2020) to avoid possible physicochemical interactions. Nominal concentrations for each assay (Table 1) were obtained via serial dilution and analytically verified by randomly taking three replicate samples from the control, the lowest as well as the highest test concentration of the feeding activity and from all test concentrations of the food choice assay. Furthermore, fresh and 3-day old test medium samples from the long-term assay were randomly taken from one replicate of each test concentration at day 0, 6, 12, 18 and day 3, 9, 15, 21, respectively. Samples were conserved at −20 °C and analyzed by using ultra-high-performance liquid chromatography-mass spectrometry (Thermo Fisher Scientific, Bremen, Germany) and quantified via external standard calibration (cf. Zubrod et al., 2015c). As only one measured AZO and CIP concentration deviated slightly more than 20 % (−20.6% and −24.0 %) from the nominal concentrations (Table S1), we consider it defensible to base the present study on the latter.

Experimental setups

The experimental procedures, including the collection of microbial and invertebrate test organisms are detailed elsewhere (see Table 1). Briefly, for the first experiment (Fig. 1a), leaf discs were cut from unconditioned black alder (*Alnus glutinosa*) leaves (hand-picked in October 2015 near Landau, Germany). Subsequently, leaves were conditioned in 12 L of conditioning medium (Dang et al., 2005) together with 50 g (wet mass) of microbial inoculum at 16 ± 1°C in darkness (hereafter called “laboratory conditions”). After 10 days, leaf discs were autoclaved, dried, weighed to the nearest 0.01 mg and re-soaked in amphipod culture medium (SAM-5S; Borgmann, 1996) for 48 h. Subsequently, male gammarids (body length of 6 – 8 mm) were fed with two pre-weighed leaf discs for 7 days that had been exposed to one of six increasing test concentrations via the water phase (Table 1; Fig. 1a). The assay was conducted under laboratory conditions and continuous aeration. Additionally, five replicates without test organisms accounted for unintended leaf mass loss by microorganisms and

handling. At test termination, dead gammarids were recorded and surviving animals as well as leaf disc remains were dried and weighed as described above.

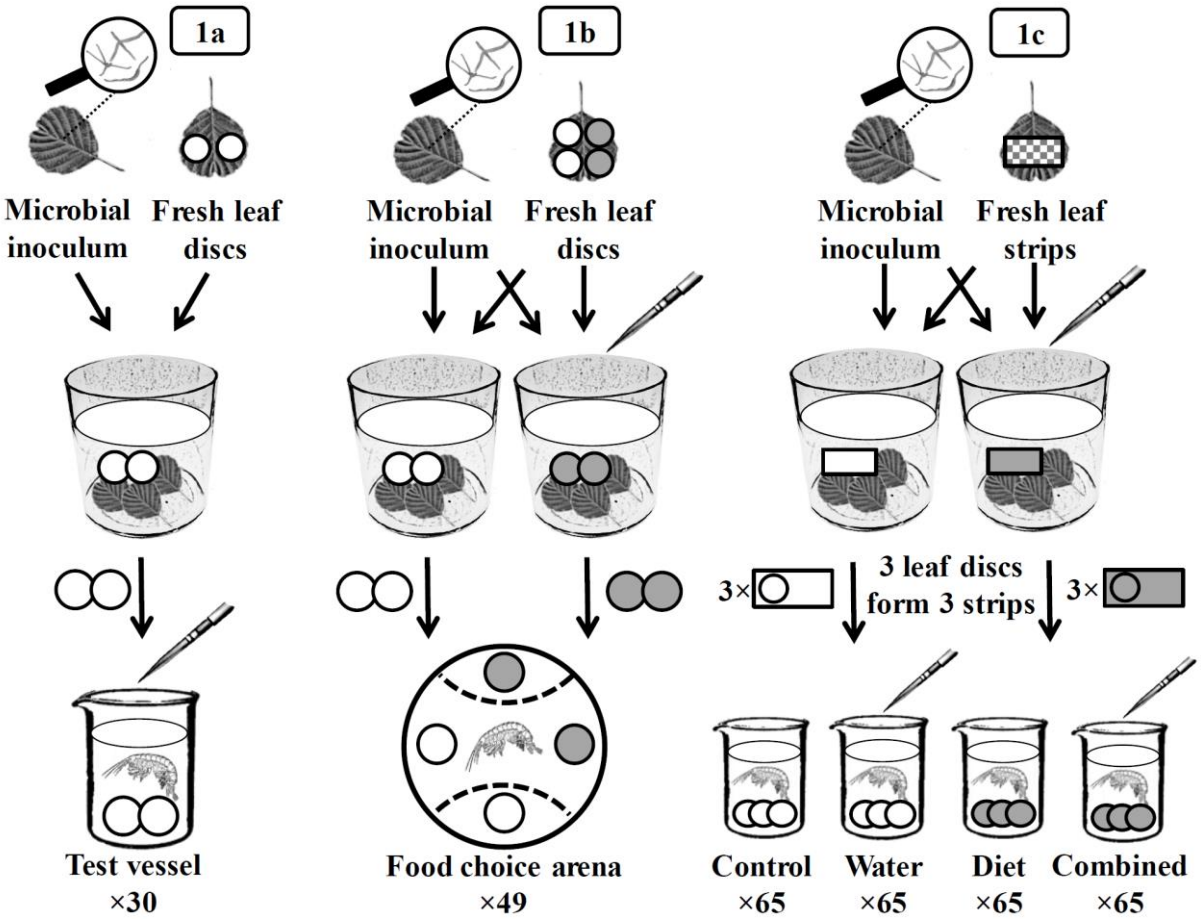

Fig. 1 Schematic overview of the three assay designs (1a, 1b and 1c; following Konschak et al., 2020). Before the start of each assay, leaf discs and strips, respectively, cut from fresh black alder leaves were microbially conditioned (by using colonized leaves with a near-natural community, i.e., microbial inoculum) in the absence and presence (denoted by the pipette) of the binary antimicrobial mixture. **1a** shows the test design of the 7-day feeding activity assay where gammarids were exposed to the antimicrobial mixture via the water phase (denoted by the pipette). **1b** displays the 24-hour food choice assay where the amphipod shredders were offered leaf discs microbially conditioned in the absence or presence of the antimicrobial mixture (denoted by white and grey discs, respectively). **1c** displays the 2 × 2-factorial study design of the 24-day long-term assay where the first factor was the absence or presence of the antimicrobial mixture in the water phase (denoted by the absence or presence of the pipette). The second factor constituted leaf discs as food source for the gammarids, which were microbially conditioned in the absence or presence of the antimicrobial mixture (denoted by white and grey discs, respectively).

For the food choice assays (Fig. 1b), sets of four leaf discs were cut from the same unconditioned black alder leaf (collected in October 2016) and dried and weighed as described above. Two leaf discs were microbially conditioned in the presence of one of four

test concentrations (treatment; n = 7; Table 1), while the two corresponding discs from the same leaf were conditioned in the absence of the antimicrobial mixture (control; Fig. 1b). Microbial conditioning was performed under laboratory conditions in 4 L of conditioning medium using 10 g (wet mass) of microbial inoculum for 12 days. Every third day, the conditioning medium with the respective test concentration was renewed to guarantee a continuous antimicrobial exposure. After 12 days, leaf discs were rinsed for ~30 min in control medium and subsequently transferred into the food choice arenas. Each arena consisted of a crystallization dish filled with SAM-5S, one male gammarid (6 – 8 mm) and the four conditioned leaf discs originating from the same leaf (Fig. 1b). The gammarid was offered one control and one treatment leaf disc, while the corresponding leaf discs of the same set were inaccessible for the animal. The inaccessible discs were used for the quantification of microbial leaf litter decomposition over the entire assay duration of 13 days. After 24 h, surviving gammarids (excluding those that had escaped from the arena) and leaf disc remains were dried and weighed as described above.

For the long-term assay (Fig. 1c), leaf strips were cut from unconditioned black alder leaves (collected in October 2017), dried, weighed and conditioned (as described for the feeding activity assay) in absence or presence of the antimicrobial mixture (n = 3; Table 1). At the end of the microbial conditioning, three pairs of leaf discs were cut from three leaf strips and directly transferred to the respective experimental units. Each replicate consisted of a 250-mL glass beaker containing 200 mL of SAM-5S, a cylindrical and a rectangular stainless steel mesh cage (mesh size = 0.5 mm), a watch glass, one male gammarid (6 – 8 mm) and six leaf discs originating from three leaf strips (see Zubrod et al., 2015b for a graphic illustration). The gammarid was kept within the cylindrical cage together with three leaf discs from different strips, while the corresponding leaf discs from the same strips were kept in the rectangular cage located at the bottom of the test vessels. The latter were protected from gammarids and were used for the determination of the microorganism- and handling-mediated leaf mass loss. During the assay, SAM-5S and leaf discs were renewed every 3 and 6 days, respectively. The 3-day old SAM-5S was filtered through a pre-weighed glass fiber filter (GF/6, Whatman, Dassel, Germany) to quantify the feces production of gammarids (Zubrod et al., 2015b) and leaf disc remains were dried and weighed to determine the leaf consumption of gammarids. At the end of the assay, surviving animals were shock-frozen in liquid nitrogen and stored at −80 °C before being lyophilized, weighed and analyzed for NLFAs.

Microbial analyses

The microbial parameters (fungal biomass, bacterial density and hyphomycete composition) were analyzed to allow for the interpretation of diet-related effects. As part of the food choice and long-term assays, 15 leaf discs of each conditioning aquarium (in total 35 and 24 samples, respectively) were analyzed for ergosterol (a proxy for fungal biomass) according to Gessner (2005). Furthermore, three leaf discs were used to determine bacterial density through fluorescence microscopy according to Buesing (2005). The community composition of aquatic hyphomycetes, a pivotal fungal group for microbial conditioning (Bärlocher, 1985), was analyzed through their spore morphology according to Pascoal and Cássio (2004).

Ergosterol from freeze-dried and weighed (to the nearest 0.01 mg) leaf material was extracted via 10 mL of a KOH-methanol, purified by using a solid-phase extraction (Sep-Pak® Vac RC tC18 500 mg sorbent, Waters, Milford, US-MA) and subsequently eluted with isopropanol (see Gessner, 2005 for more details). Afterwards, ergosterol concentrations were determined by high-performance liquid chromatography with UV-vis detection (1200 Series, Agilent Technologies, Santa Clara, US-CA) and a LiChrospher® 100 RP-18 column (250 mm × 4.6 mm, particle size 5 µm, Merck Millipore, Billerica, US-MA) and via external standard calibration curve. Ergosterol was normalized to leaf dry mass.

To determine bacterial densities, cells preserved in a 2 % formaldehyde/0.1 % sodium pyrophosphate solution were detached from leaf discs by ultrasonication. An aliquot of the cell suspension (10 µL) was filtered (pore size 0.2 µm; AnodiscTM 25, Whatman, Maidstone, UK) and bacteria were stained with SYBR® Green II (Molecular Probes, Eugene, US-OR). Cells were quantified by fluorescence microscopy involving the software AxioVision (Axio Scope.A1, AxioCam MRm and AxioVision Rel. 4.8, Carl Zeiss MicroImaging, Jena, Germany; see Buesing, 2005 for more details). Bacterial cell counts were normalized to leaf dry mass.

To analyze the hyphomycete community composition, five leaf discs per aquarium were shaken (120 rpm) for 96 h in deionized water at 16 ± 1°C. An aliquot of the deionized water (10 mL), containing fungal spores, was filtered (S-Pak Filters 0.45 µm, 47 mm white gridded, Merck Millipore, Billerica, US-MA) and spores were stained by cotton blue. Spores were identified by using various identification keys (e.g., Ingold, 1975). Spore counts were normalized to leaf dry mass.

Fatty acid analyses

FAs and NLFAs of microbially conditioned leaves and gammarids, respectively, originating from the long-term assay were analyzed to investigate microorganism-mediated food quality effects on *G. fossarum*. Five leaf strips and ten gammarids per aquarium and treatment (in total 24 and 40 samples) were lyophilized and weighed as described above. Total FAs of leaves and NLFAs of gammarids were quantified according to Fink (2013) and Konschak et al. (2020), respectively.

Leaves were freeze-dried, manually crushed and weighed to the nearest 0.01 mg. A chloroform-methanol mixture (2:1) as well as an internal standard (Tristearin-D105, Larodan, Solna, Sweden) was added and lipids and FAs were extracted overnight at −20 °C. Hydrolysis of lipids and derivatization of FAs to fatty acid methyl esters (FAMEs) was obtained via 3N methanolic HCl (Sigma-Aldrich, St. Louis, US-MO). FAMEs were extracted via liquid-liquid extraction using isohexane (see Fink, 2013 for more details), a procedure that increases the purification of leaf samples compared to the derivatization with trimethylsulfonium hydroxide (TMSH; see below). FAMEs were measured by using gas chromatography with flame ionization detector (GC; CP-3800, Varian, Palo Alto, US-CA) and a DB-225 GC column (30 m, ID 0.25 mm, film thickness 0.25 µm, J&W Scientific, Agilent Technologies, Santa Clara, US-CA; cf. Fink, 2013). The carrier gas was nitrogen. The identification of FAMEs is based on retention times of standards (Sigma-Aldrich, St. Louis, US-MO). Concentrations of FAs were quantified using external standard calibration curves and were corrected via extraction blanks and the recovery rate of the internal standard. Corrected FA concentrations were extrapolated to the sample volume and normalized to sample dry mass.

Gammarids were homogenized in a chloroform-methanol-water mixture (1:2:0.8) via Ultra-Turrax blender at 6500 rounds/min for few seconds (T25 basic, IKA® Werke GmbH & Co. KG, Staufen, Germany). The internal standard (see above) was added and the homogenate was stored in a chloroform-methanol-water mixture (2:2:1.8) overnight at 4°C. Neutral lipids were separated via solid-phase extraction (Chromabond® easy polypropylene columns, Macherey-Nagel, Düren, Germany) and NLFAs were rapidly transesterified with TMSH (Sigma-Aldrich, St. Louis, US-MO; see Konschak et al., 2020 for more details). FAMEs were measured and NLFAs were quantified as described above.

Calculations and endpoint estimations

Leaf consumption (in mg leaf material/mg gammarid/day) during the feeding activity and food choice assays was calculated as described by Naylor et al. (1989) and Bundschuh et al. (2009), respectively. Microbial leaf decomposition (in mg leaf mass loss/day), determined during the food choice assay, was quantified according to Zubrod et al. (2015a). During the long-term assay, leaf consumption and feces production of *G. fossarum* (both in mg/day) were calculated as per Zubrod et al. (2011). Growth (in µg gammarid dry mass gain/day) was calculated by subtracting the median dry mass of 70 animals (shock-frozen at the beginning of the assay) from the final dry mass of each surviving gammarid at the termination of the assay divided by the study duration in days (i.e., 24). Animals that died during the assays were excluded from any data evaluation. The EC_{20} and EC_{50} values based on leaf consumption of *G. fossarum* measured during the feeding activity assay were calculated by fitting a series of concentration-response models to the data. The dose-response curve of Cedergreen et al. (2005) was the best fitting model (based on Akaike's information criterion) to the feeding activity data (Table S2).

Independent action model

Expected joint effects of the binary antimicrobial mixture on all gammarid-related endpoints measured during the bioassays (leaf consumption, food selection, feces production and growth) as well as on microbial leaf decomposition, were calculated according to the IA model (Bliss, 1939):

$$E_{Mix} = E_{AZO} + E_{CIP} - E_{AZO} \times E_{CIP}$$

where E_{Mix} is the predicted mixture effect (ranging from 0 to 1) based on the individual effects of each component (E_{AZO} and E_{CIP}, respectively) when acting alone on the test organism(s) at the concentration present in the mixture. Individual mixture component effects (E_{AZO} and E_{CIP}, respectively, ranging from 0 to 1) were calculated as follows:

$$E_{AZO/CIP} = 1 - \frac{T_i}{C}$$

where T_i is the value of the response variable at concentration i and C is the value of the respective control. Due to the high natural variability of the measured response variables in the present study, a minimal effect threshold of 20 % for effects unrelated to statistical significance was chosen to avoid an overestimation of IA predictions (i.e., E_{AZO} and E_{CIP}

below 0.2 were set to zero). This threshold was selected, since a 20 % effect is considered environmentally relevant (Bruce and Versteeg, 1992; Peters et al., 2013), which is still an acceptable effect size for populations of aquatic species (Barnthouse et al., 2008). The compliance of predicted with observed effects was concluded, if point estimates fell within the 95 % confidence intervals of the observed mean or median effects. Otherwise, interaction effects (i.e., synergism and antagonism) were assumed. Since leaf-associated microbial communities and (NL)FA levels are highly variable between different seasons (Nikolcheva and Bärlocher, 2005; Guo et al., 2018), effect predictions were not determined for microbial parameters and (NL)FAs.

Statistical analyses

Prior to applying statistical tests, extreme values were identified via visual inspection of boxplots (with a 1.5 × interquartile range) and excluded from further analyses. Data were tested for normal distribution via quantile-quantile plots as well as Shapiro-Wilk test and were checked for variance homogeneity by using residual plots and Levene's test. Parametric unpaired data containing one factorial predictor with two factor levels and at least three factor levels (i.e., microbial parameters and FAs of conditioned leaves) were evaluated via Student's *t*-test and one-way ANOVA followed by Dunnett's test, respectively. Non-parametric unpaired data (i.e., microbial parameters and leaf consumption of gammarids) were analyzed using Wilcoxon rank-sum test followed by a Bonferroni correction for multiple comparisons if necessary (i.e., ≥ three factor levels). Parametric and non-parametric paired data with one factorial predictor and two factor levels (i.e., food selection and microbial decomposition) were analyzed via paired *t*-tests and Wilcoxon signed-rank tests, respectively. Parametric and non-parametric unpaired data consisting of two factorial predictors and two factor levels (i.e., microbial parameters as well as energy processing, growth and NLFAs of gammarids) were evaluated using two-way analysis of variance (ANOVA) and rank-transformed two-way ANOVA, respectively. Multivariate data were square-root transformed to reduce the discriminatory power of prevalent hyphomycete species and (NL)FAs, respectively. Subsequently, data were checked for dispersion effects before testing for location effects via permutational multivariate analysis of variance (PERMANOVA). More details of null hypothesis significance tests (NHSTs; i.e., sum and mean of squares, *F*-statistics and *p*-values) and group medians with 95 % confidence intervals for each response variable are listed in Tables S3 – S7. Moreover, data distribution for leaf consumption, food selection, feces production and growth of gammarids as well as for leaf decomposition is displayed in

Fig. S1 – S3. NHST, dose-response modeling and figures were performed using R Version 3.5.1 for Windows (R Core Team, 2014) in combination with the add-on packages (*asbio*, *drc*, *multcomp*, *plotrix* and *vegan*). Note that "statistically significant" (i.e., the *p*-value < α of 0.05) is abbreviated with "significant" throughout the entire study.

Results

During the feeding activity assay, a non-significantly increased leaf consumption of gammarids (by ~30 %) was observed at the lowest test concentration. This response variable was reduced with increasing antimicrobial mixture concentration (up to ~60 %; Fig. 2). Predictions of the IA model were within the 95 % confidence intervals of mean observed effects and the fitted concentration-response curve with exception of the lowest test concentrations (difference of ~40 %; Fig. 2).

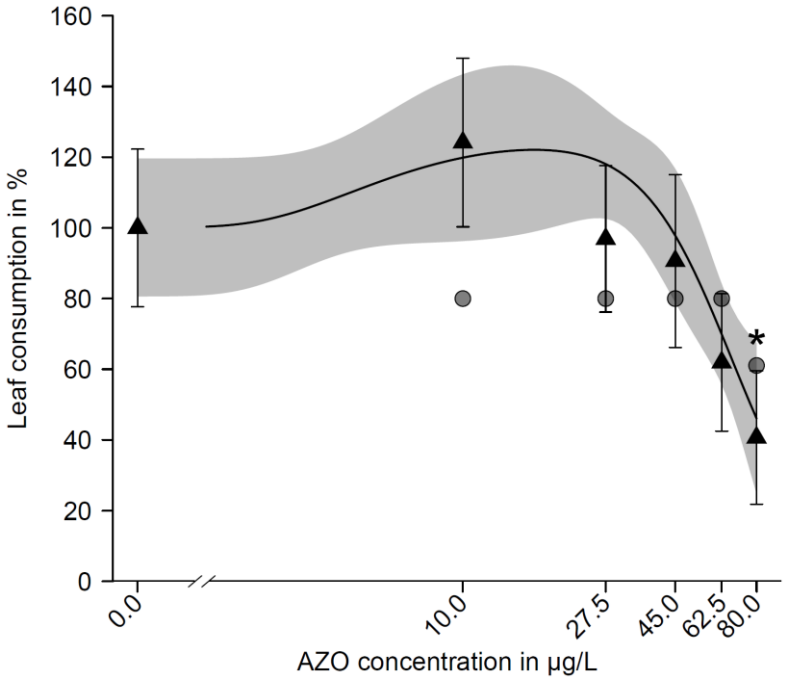

Fig. 2 Mean (with ± 95 % CI) percentage effect on the leaf consumption of gammarids (black triangles) when the animals were subjected to the binary antimicrobial mixture with increasing AZO concentrations and a fixed CIP concentration of 500 µg/L. Moreover, the model with the best fit (black line with ± 95 % CI) and IA predictions (grey circles) derived from the feeding activity assays, where the mixture components were tested individually, are displayed. The asterisk indicates a statistically significant difference to the control.

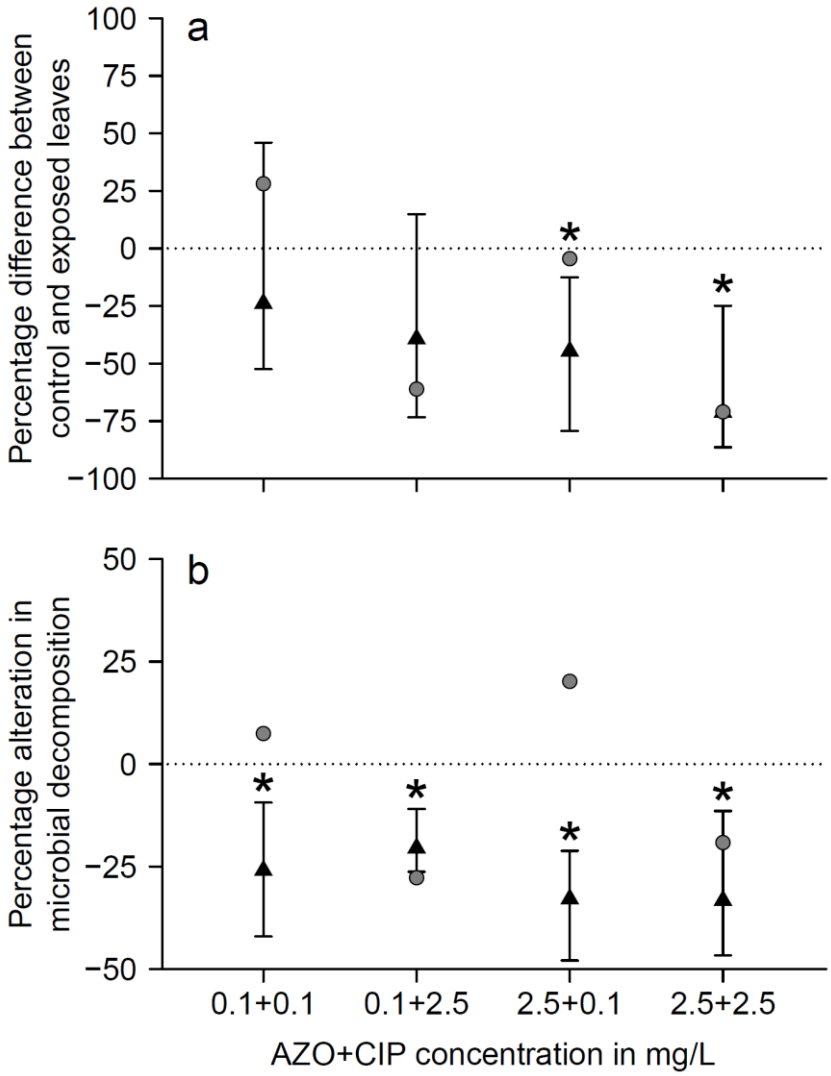

Fig. 3 Median (with ± 95 % CI) percentage effect (relative to the respective control) of (**a**) food selection of gammarids and (**b**) microbial leaf decomposition (black triangles) when subjected to different concentrations of the binary antimicrobial mixture. Furthermore, IA predictions derived from the food choice assays with the individual tested mixture components are displayed as grey circles. Asterisks indicate a statistically significant difference to the control.

During the food choice assay, the gammarids non-significantly preferred control leaf discs over those discs conditioned in the presence of 0.1 + 0.1 and 0.1 + 2.5 mg/L AZO + CIP, while a significant preference was observed for control leaf discs over those which were exposed to the antimicrobial mixtures containing 2.5 mg/L AZO (Fig. 3a; Table S4). Each effect prediction fell within the 95 % confidence interval of the respective observed median effect, except for one treatment (i.e., 2.5 + 0.1 mg/L AZO + CIP; difference of ~40 %; Fig. 3a). Microbial leaf decomposition and hyphomycete community composition were significantly negatively affected by all antimicrobial mixtures compared to the control (Fig.

3b, Table S4). The relative mean contribution of *Tetracladium marchalianum* to fungal sporulation increased in the presence of the antimicrobial mixture, while the share of all other species decreased (Fig. S4). Furthermore, AZO and CIP significantly reduced fungal biomass and bacterial densities, respectively, while both antimicrobials significantly affected fungal sporulation (Table S5). The IA model predictions for microbial leaf decomposition matched the observed effects in presence of 2.5 mg/L CIP, while predictions did not match the observed effects when leaf discs were microbially conditioned in presence of 0.1 mg/L CIP (i.e., 0.1 + 0.1 and 2.5 + 0.1 mg/L AZO + CIP; difference of ~30 % and ~50 %; Fig. 3b).

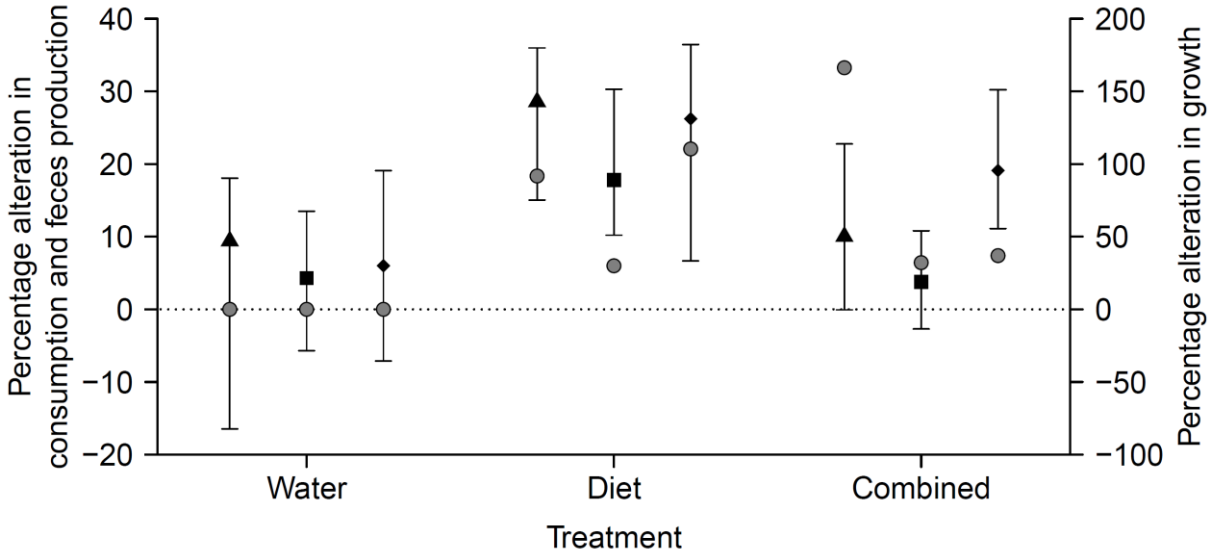

Fig. 4 Median (with ± 95 % CI) percentage effect (relative to the control) on the leaf consumption of gammarids (black triangles), feces production (black squares) and growth (black diamonds) when the animals were subjected to different effect pathways during the long-term assay with binary antimicrobial mixtures. IA predictions derived from the long-term assays with the individual tested mixture components are displayed as grey circles.

During the long-term assay, no waterborne effects were observed, while 15 + 500 µg/L AZO + CIP applied via the dietary pathway significantly increased the energy processing and growth as well as non-significantly elevated the NLFA content (by ~50 %) of *G. fossarum* compared to the control (Fig. 4, Table S6). When both pathways acted jointly, effect sizes of all response variables were lower compared to the sum of the effects induced by each pathway individually (Fig. 4; Table S6). Moreover, no effects on leaf quality related parameters (i.e., fungal biomass, aquatic hyphomycete community composition and FAs associated with conditioned leaves) were observed when conditioned in presence of the antimicrobial mixture (Table S7). The IA model predictions were within the 95 % confidence

intervals of median observed effects for the individual pathways, except for feces production in the Diet treatment (difference of ~10 %; Fig. 4). However, except for feces production, the IA model predictions for leaf consumption and growth did not match the median observed effects (difference of ~20 % and ~60 %; Fig. 4) when both pathways acted jointly (i.e., Combined treatment).

Discussion

Short-term waterborne effects

As expected, effect predictions of the IA model mirrored observed effects of the binary antimicrobial mixture on the leaf consumption of gammarids, except for the lowest tested concentration (Fig. 2). The stimulated leaf consumption at 10 + 500 µg/L AZO + CIP indicates a hormetic effect at lower mixture concentrations, which had not been observed in previous studies where the mixture components had been applied individually (Zubrod et al., 2014; Konschak et al., 2020). This stimulation might be explained by a higher energy demand induced by chemical stress, which consequently resulted in an increased energy intake (e.g., Eriksson-Wiklund et al., 2011). Even though meaningful reductions in leaf consumption occurred at concentrations of the antimicrobials below or equal to the EC_{20} of the individual components in the antimicrobial mixture, no synergistic effects could be confirmed, suggesting that the IA model reflects the risk imposed by waterborne exposure for *G. fossarum*.

Effects on food selection and microbial decomposition

Contrary to our hypothesis, predictions largely matched the observed food choice of gammarids (Fig. 3a). Such an accuracy of the IA model predictions could, however, not be reached when mixture components induced effects in opposite directions, when applied individually. This is the case for the mixture of 2.5 + 0.1 mg/L AZO + CIP: 2.5 mg/L AZO resulted in a preference of *G. fossarum* for control leaves (Zubrod et al., 2015a), while gammarids tended to prefer leaves conditioned in presence of 0.1 mg/L CIP (Konschak et al., 2020). The IA model predicted that the effects of both antimicrobials should cancel each other out when applied as a mixture. Our observations, however, suggest a further reduction in leaf palatability for gammarids relative to the presence of AZO alone. Leaf palatability mainly depends on leaf conditioning by leaf-associated aquatic fungi (Bärlocher, 1985).

Consequently, the significantly affected fungal biomass associated with leaf litter exposed to this antimicrobial mixture could explain the observed food selection pattern.

Similar to food selection, model predictions did not comply with the observed mixture effects on microbial leaf decomposition when the individual mixture components induced effects in opposite directions (Fig. 3b). When only CIP was present at 0.1 mg/L, it stimulated microbial leaf decomposition (Konschak et al., 2020), whereas AZO (at either 0.1 and 2.5 mg/L) reduces this response variable (Zubrod et al., 2015a). While these changes have been linked to shifts in the aquatic hyphomycete communities, the effects of the antimicrobial mixture on microbial leaf decomposition could not solely be explained by alterations in the aquatic hyphomycete composition, but also by impairments in microbial sum parameters (i.e., fungal biomass and bacterial density; Table S4). Moreover, it is alarming that the lowest mixture concentration (0.1 + 0.1 mg/L AZO + CIP) unexpectedly caused effects on the microbial leaf decomposition comparable in magnitude to the highest tested concentration (2.5 + 2.5 mg/L AZO + CIP; Fig. 3b). This indicates a steeper concentration-response course of the antimicrobial mixture compared to the individual mixture components. Therefore, further investigations targeting the impact of complex antimicrobial mixtures at lower and, thus, field relevant concentrations on natural microbial communities and their functions are urgently needed.

Long-term waterborne and dietary effects

In line with our hypothesis, the IA model predictions comply with observed waterborne mixture effects in *G. fossarum* during the long-term assay (Fig. 4). While the IA model predictions for leaf consumption and growth matched the observed effects of gammarids in the Diet treatment, the deviation between expected and observed effects on feces production indicates a slight synergistic action (Fig. 4). This might be related to the observed increase in leaf consumption, which deviated by 10 % from the IA prediction. This increased energy intake was most likely induced by CIP (cf. Konschak et al., 2020), resulting in turn in an increased growth and NLFA content. Konschak et al. (2020) suggested a stimulation of the leaf consumption of gammarids by CIP-induced alterations of the microorganism-mediated food quality. However, contrary to their study, no alterations in microbial parameters were observed in the present study (Table S7). An alternative explanation for the observed effects in *Gammarus* might be a CIP-induced alteration in the gut microbiome or the immune system of gammarids as both can have an impact on the energy processing and behavior of animals (Brown et al., 2017). Since neither the gut microbiome nor the immune system of

invertebrates are well understood (Loker et al., 2004; Lee and Hase, 2014), further studies are needed to draw final conclusions about mechanisms underlying the observed effects.

When the waterborne and dietary effect pathway of the binary antimicrobial mixture acted jointly during the 24-day bioassay, the observed effects on the leaf consumption of gammarids were overestimated by the IA model prediction, indicating antagonism (i.e., the observed effect is lower than expected). In contrast, the IA model underestimated the growth of gammarids, indicating positive synergistic interactions (Fig. 4). As the microbial parameters did not indicate bottom-up effects on *G. fossarum*, the different responses may be explained by an altered uptake of AZO and CIP when gammarids were exposed to the mixture instead of the individual substances. However, to our best knowledge, no studies exist that investigated differential uptake rates of strobilurins and quinolones in animals when applied simultaneously. Therefore, analyses of internal concentrations of both substances in gammarids (when applied individually and in mixture) may help to shed light on the mechanisms explaining the differential responses in the amphipod shredder. Furthermore, future studies should incorporate additional leaf-associated microorganisms (e.g., algae; Crenier et al., 2017) that are known to influence energy processing and physiology of shredders to reveal further, potentially overlooked bottom-up effects.

The results of the present study show that the IA model accurately predicts the effects of AZO and CIP on amphipod shredders when focusing on single effect pathways. In contrast, at the leaf-associated microbial community level and when the waterborne and the dietary pathway acted jointly on gammarids, the effect predictions were inaccurate, resulting partly in over- or underestimations of adverse effects. These insights highlight the need for more studies incorporating ecological concepts (e.g., horizontal and vertical species interactions) that uncover indirect effects and help to develop a more holistic picture of the risks associated with the unintended release of antimicrobials into surface waters.

Conclusion

We demonstrate that microbial decomposers and shredders can show unexpected effect patterns in the presence of antimicrobial mixtures when data of the individual components of the mixture were used to derive mixture effect predictions. Even though some of the concentrations assessed in the present study were beyond field relevance, direct and indirect effects of fungicides and antibiotics at environmental concentrations have been reported elsewhere (e.g., Bundschuh et al., 2017; Zubrod et al., 2017). When antimicrobials occur

simultaneously in surface waters at sublethal effect levels (e.g., by entering aquatic systems via wastewater treatment plants; Batt et al., 2006; LUWG, 2011), unpredicted mixture effects seem possible. Moreover, antimicrobial mixture effects in aquatic ecosystems may intensify in the future, since an increasing fungicide as well as antibiotic use is forecasted (Elad and Pertot, 2014; Klein et al., 2018). In the light of the expected intensification of antimicrobial mixtures in the environment, studies investigating the processes and mechanisms on ecosystem level are urgently needed to inform environmental authorities about the potential risks of antimicrobial substances in the environment.

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Supporting information of Appendix A3

Distribution and probability density of percentage leaf consumption, food selection, feces production and growth of gammarids as well as microbial leaf decomposition (Fig. S1 – S3)

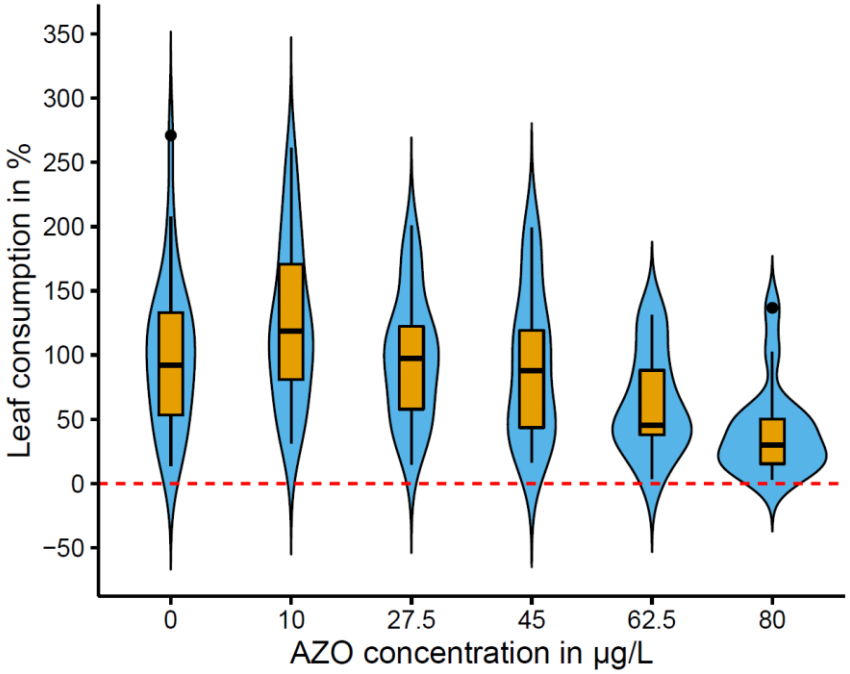

Fig. S1 Blue violin plots combined with yellow boxplots (25, 50, 75 percentiles and 1.5 × interquartile range) show distribution and probability density of the percentage effect on the leaf consumption of gammarids when the animals were subjected to the binary antimicrobial mixture with increasing AZO concentrations and a fixed CIP concentration of 500 µg/L during the feeding activity assay.

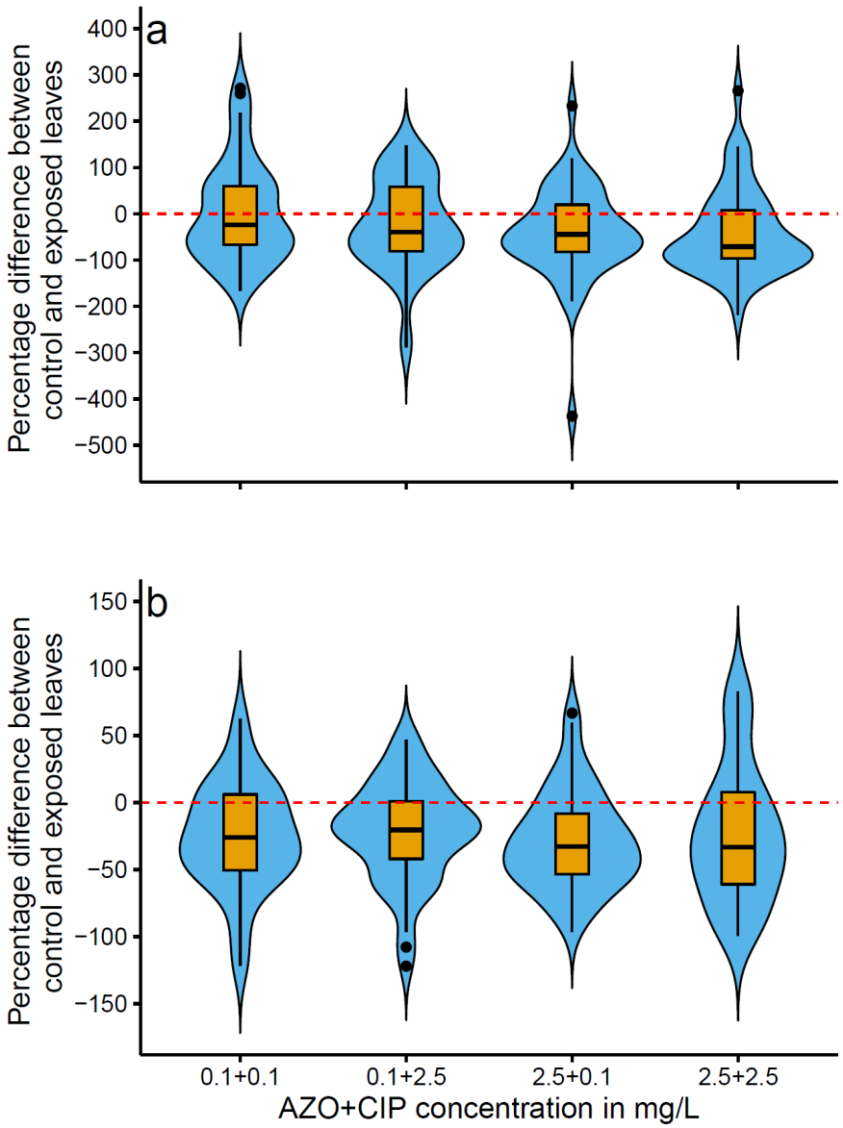

Fig. S2 Blue violin plots combined with yellow boxplots (25, 50, 75 percentiles and 1.5 × interquartile range) show distribution and probability density of the percentage effect (relative to the respective control) of (a) food selection of gammarids and (b) microbial leaf decomposition when subjected to different concentrations of the binary antimicrobial mixture during the food choice assay.

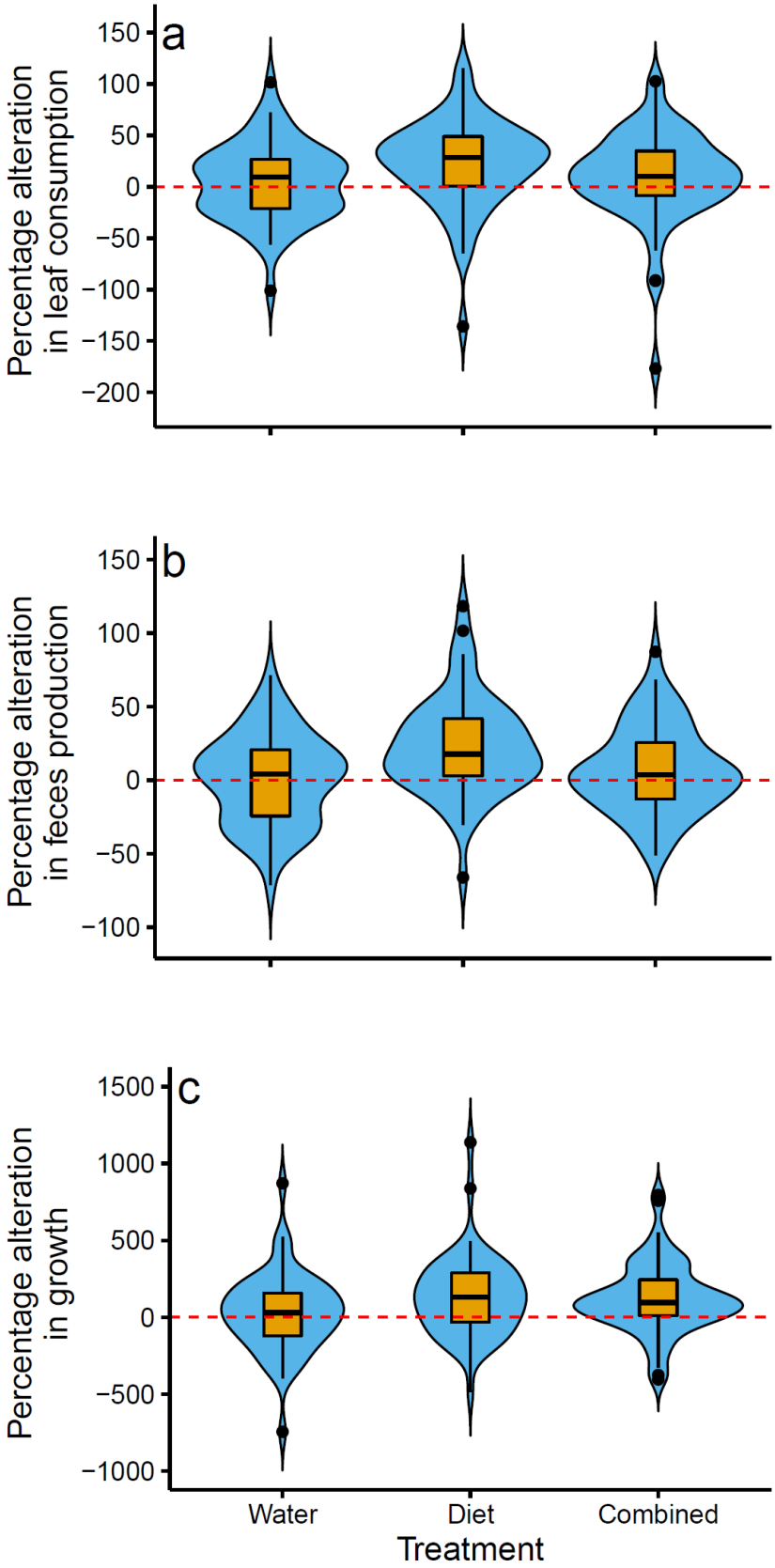

Fig. S3 Blue violin plots combined with yellow boxplots (25, 50, 75 percentiles and 1.5 × interquartile range) show distribution and probability density of the percentage effect (relative to the respective control) on (a) leaf consumption, (b) feces production and (c) growth of gammarids when the animals were subjected to different effect pathways during the long-term assay with binary antimicrobial mixtures.

Aquatic hyphomycete communities during the food choice assay

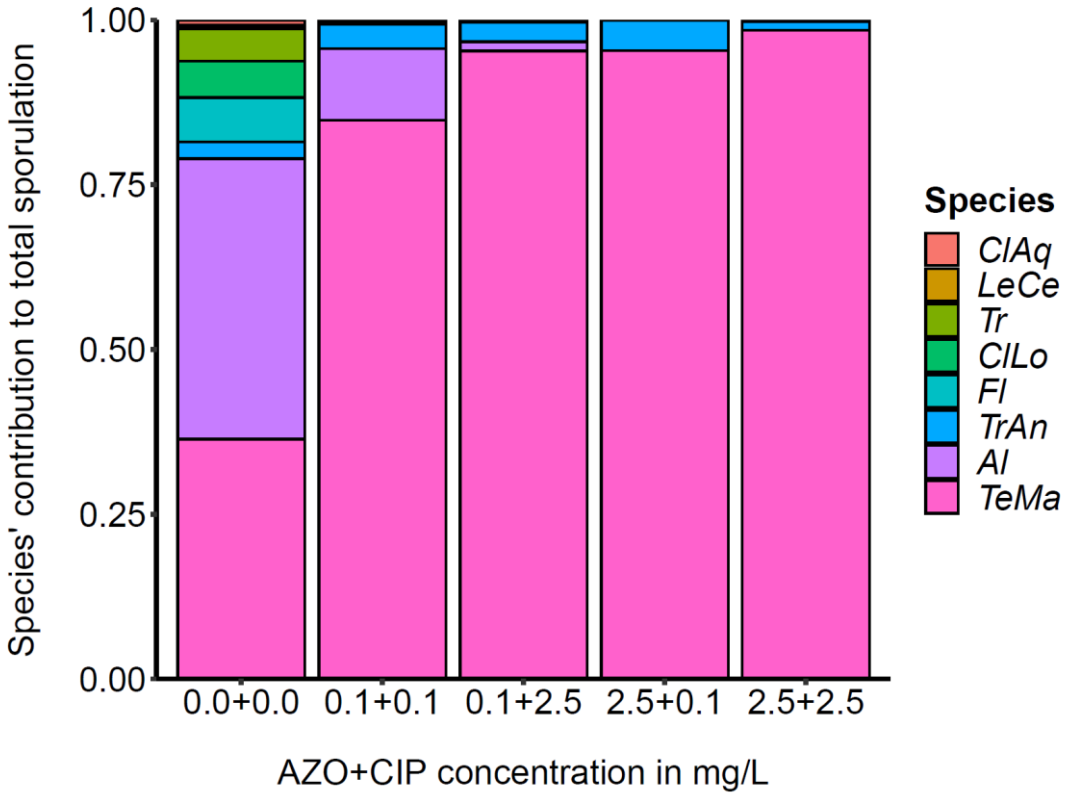

Fig. S4 Mean contribution of species to total aquatic hyphomycete sporulation on leaf discs conditioned in the presence of different mixture concentrations during the food choice assay. Note that each color scheme is aligned to the species order of the control with following order: *Tetracladium marchalianum* (*TeMa*), *Alatospora* sp. (*Al*), *Tricladium angulatum* (*TrAn*), *Flagellospora* sp. (*Fl*), *Clavatospora longibrachiata* (*ClLo*), *Tricladium* sp. (*Tr*), *Lemonniera centrosphaera* (*LeCe*) and *Clavariopsis aquatica* (*ClAq*).

Neutral lipid fatty acids (NLFAs) of gammarids during the long-term assay

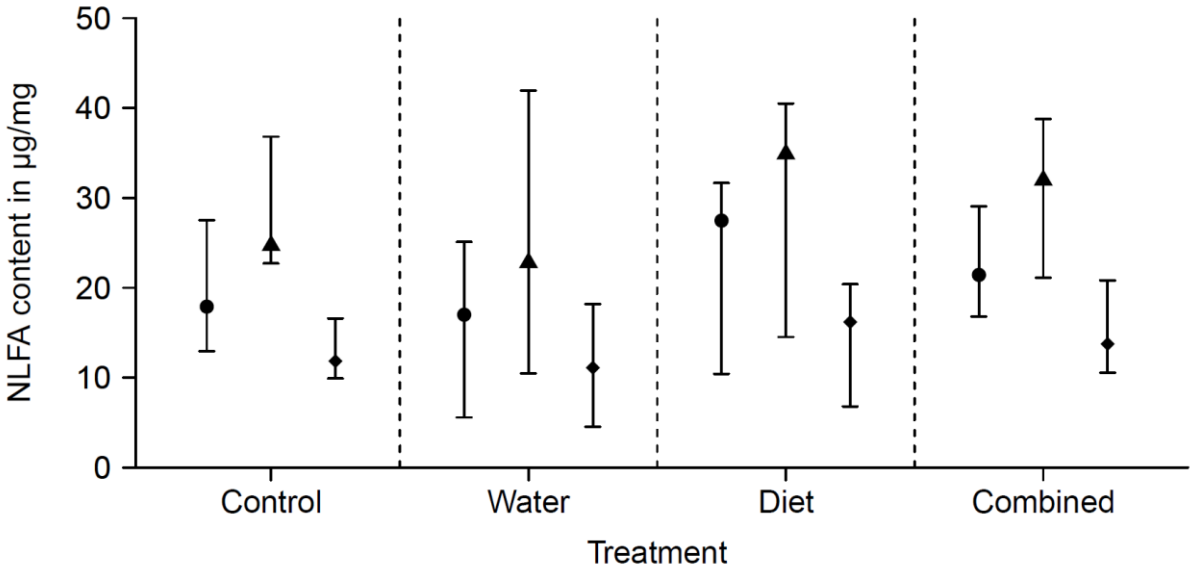

Fig. S5 Median (with ± 95 % CI) saturated (SAFA; points), monosaturated (MUFA; triangles) and polysaturated (PUFA; diamonds) fatty acid content of *G. fossarum* subjected to different treatments during the 24-day long-term assay with the binary antimicrobial mixture (i.e., 15 + 500 µg/L AZO+CIP). Statistical analyses are displayed in Table S6.

Results of chemical analyzes

Table S1 Nominal and measured (mean with ± 95 % CI) concentrations of mixture components (i.e., AZO and CIP) for the respective assays with the respective lowest calibration level (LCL).

Assay	Test medium	Treatment	Substance	LCL in µg/L	Nominal in µg/L	Fresh medium in µg/L	3-day-old medium in µg/L
Feeding	SAM-5S[a]	Control	AZO	0.50	0	<LCL	-
activity		AZO + CIP			10	10.39 ± 1.74	-
					80	81.26 ± 14.75	-
		Control	CIP	1.00	0	<LCL	-
		AZO + CIP			500	442.83 ± 38.41	-
Food choice	Conditioning[b]	Control	AZO	0.50	0	<LCL	-
		AZO + CIP			100	87.10 ± 4.62	-
					2500	1985.63 ± 112.29	-
		Control	CIP	0.50	0	<LCL	-
		AZO + CIP			100	91.95 ± 8.60	-
					2500	2132.87 ± 199.07	-
Long-term	Conditioning[b]	Control	AZO	0.50	0	<LCL	-
		AZO + CIP			15	15.36 ± 2.48	-
		Control	CIP	0.50	0	<LCL	-
		AZO + CIP			500	380.18 ± 30.56	-
	SAM-5S[a]	Control	AZO	0.50	0	<LCL	-
			CIP	0.50	0	<LCL	-
		Water	AZO		15	16.34 ± 1.11	13.41 ± 0.87
			CIP		500	522.07 ± 32.12	276.62 ± 69.21
		Diet	AZO		-	-	1.20 ± 0.50
			CIP		-	-	12.07 ± 3.54
		Combined	AZO		15	SeeWater	-
			CIP		500	SeeWater	-

[a]Culture medium according to Borgmann (1996)

[b]Microbial conditioning medium according to Dang et al. (2005)

Concentration-response models for feeding activity data

Table S2 Models used for concentration-response modeling and their respective coefficients for the feeding activity assay.

Endpoint	Model	Parameters[a]			
Leaf consumption	Cedergreen-Ritz-Streibig (with 4 parameters)	b=2.04	d=100.12	e=60.81	f=27.04

[a]Parameterization according to Ritz and Streibig (2005) and Cedergreen et al. (2005)

Results of statistical data analyses for each assay

Table S3 Mortality of gammarids and group medians (with ± 95 % CI) for leaf consumption of the feeding activity assay (n = 30). Moreover, statistical tests used as well as p-values from statistical comparisons of the binary mixture treatments with the control (p-values below 0.05 are printed in bold) and effect concentrations resulting in 20 and 50 % of mortality and inhibition of leaf consumption (EC_{20} and EC_{50} values) in µg/L AZO + CIP (with ± 95 % CI) for mortality and leaf consumption are shown.

Endpoint	AZO in µg/L + 500 µg/L CIP	Mortality (%) or Median	±95 % CI	Statistical test	p-value (after Bonferroni adjustment)	EC_{20}	EC_{50}
Mortality	0.00	0.00	0.00 to 11.57	Proportion test		62.46	-
	10.00	0.00	0.00 to 11.57		1.000	(62.37 to	
	27.50	0.00	0.00 to 11.57		1.000	62.56)	
	45.00	3.33	0.08 to 17.22		1.000		
	62.50	20.00	7.71 to 38.57		0.157		
	80.00	46.67	28.34 to 65.67		**<0.001**		
Leaf	0.00	0.26	0.20 to 0.36	Wilcoxon rank-sum		46.58	74.97
consumption	10.00	0.34	0.25 to 0.45		0.768	(29.17 to	(48.59 to
	27.50	0.28	0.17 to 0.35		1.000	64.00)	101.35)
	45.00	0.25	0.13 to 0.34		1.000		
	62.50	0.13	0.11 to 0.26		0.068		
	80.00	0.09	0.04 to 0.15		**0.001**		

Table S4 Number of analyzed replicates and group medians (with ± 95 % CI) for the response variables analyzed during the food choice assay. Moreover, statistical tests used as well as *p*-values from statistical comparisons of the binary mixture treatments with the respective control are shown. All *p*-values below 0.05 are printed in bold.

Endpoint	AZO + CIP in mg/L	*n*	Median	±95 % CI	Statistical test	*p*-value
Leaf consumption in mg/mg individual/d	0.0 + 0.0	44	0.19	0.10 to 0.29	Student's *t* (paired)	
	0.1 + 0.1		0.14	0.09 to 0.28		0.646
	0.0 + 0.0	46	0.17	0.11 to 0.26		
	0.1 + 2.5		0.11	0.05 to 0.20		0.309
	0.0 + 0.0	46	0.16	0.06 to 0.26		
	2.5 + 0.1		0.09	0.03 to 0.14		**0.015**
	0.0 + 0.0	47	0.15	0.11 to 0.25		
	2.5 + 2.5		0.04	0.02 to 0.12		**0.002**
Microbial leaf decomposition in mg/d	0.0 + 0.0	45	0.13	0.10 to 0.15	Wilcoxon signed-rank	
	0.1 + 0.1		0.10	0.07 to 0.12		**<0.001**
	0.0 + 0.0	47	0.14	0.12 to 0.15		
	0.1 + 2.5		0.11	0.10 to 0.12		**<0.001**
	0.0 + 0.0	47	0.15	0.14 to 0.16		
	2.5 + 0.1		0.10	0.08 to 0.12		**<0.001**
	0.0 + 0.0	47	0.13	0.11 to 0.16		
	2.5 + 2.5		0.09	0.07 to 0.12		**<0.001**
Fungal biomass in µg ergosterol/g leaf dry mass	0.0 + 0.0	7	76.83	30.83 to 145.27	Wilcoxon rank-sum (with Bonferroni adjustment)	
	0.1 + 0.1	7	39.32	26.53 to 65.23		0.291
	0.1 + 2.5	7	66.62	24.30 to 122.10		1.000
	2.5 + 0.1	7	33.18	20.36 to 44.29		0.070
	2.5 + 2.5	7	32.72	21.40 to 59.68		0.291
Bacterial density in 10^9 cells/g leaf dry mass	0.0 + 0.0	6	1.15	0.32 to 1.77	Dunnett's *t*	
	0.1 + 0.1	6	0.64	0.40 to 1.30		0.077
	0.1 + 2.5	7	0.41	0.21 to 0.93		**0.004**
	2.5 + 0.1	7	0.75	0.45 to 1.40		0.200
	2.5 + 2.5	7	0.33	0.05 to 0.69		**<0.001**
Fungal spores/mg leaf dry mass	0.0 + 0.0	7	332.34	87.15 to 410.96	Wilcoxon rank-sum (with Bonferroni adjustment)	
	0.1 + 0.1	7	70.86	33.47 to 324.19		0.152
	0.1 + 2.5	7	212.32	58.64 to 599.71		1.000
	2.5 + 0.1	7	5.62	00.00 to 23.44		**0.002**
	2.5 + 2.5	7	63.33	40.30 to 576.25		0.212
Hyphomycete community composition	0.0 + 0.0	7	-	-	PERMANOVA (with Bonferroni adjustment)	
	0.1 + 0.1	7	-	-		**0.020**
	0.1 + 2.5	7	-	-		**0.008**
	2.5 + 0.1	7	-	-		**0.008**
	2.5 + 2.5	7	-	-		**0.004**

Table S5 ANOVA-tables for microbial response variables (n = 7) during the food choice assay. All p-values < 0.05 are printed in bold.

Endpoint	Factor	df1	SS	MS	F-value	p-value
Bacterial density in 10^9 cells/g leaf dry mass	*AZO*	1	0.1	0.1	0.003	0.956
	CIP	1	500.5	500.5	10.717	**0.003**
	AZO × *CIP*	1	63.4	63.4	1.358	0.256
	Residuals	23	1074.0	46.7		
Fungal biomass in µg ergosterol/g leaf dry mass	*AZO*	1	386.3	386.3	7.487	**0.012**
	CIP	1	185.1	185.1	3.588	0.070
	AZO × *CIP*	1	17.3	17.3	0.335	0.568
	Residuals	24	1238.3	51.6		
Fungal spores/mg leaf dry mass community composition	*AZO*	1	549.1	549.1	20.538	**<0.001**
	CIP	1	567.0	567.0	21.206	**<0.001**
	AZO × *CIP*	1	69.1	69.1	2.586	0.121
	Residuals	24	641.7	26.7		

Table S6 (PERM)ANOVA-tables for all gammarid-related response variables during the 24-day long-term assay. All *p*-values < 0.05 are printed in bold.

Endpoint	Factorial predictor	df1	SS	MS/R2	*F*-value	*p*-value	ANOVA type
Leaf consumption	*Water*	1	0.063	0.06261	1.134	0.288	Two-way
	Diet	1	0.280	0.28040	5.077	**0.025**	ANOVA
	Water × *Diet*	1	0.099	0.09891	1.791	0.182	
	Residuals	226	12.482	0.05523			
Feces production	*Water*	1	0.059	0.05881	3.526	0.062	Two-way
	Diet	1	0.224	0.22428	13.446	**<0.001**	ANOVA
	Water × *Diet*	1	0.076	0.07635	4.578	**0.033**	
	Residuals	226	3.77	0.01668			
Growth	*Water*	1	540	540	0.129	0.720	Two-way
	Diet	1	47782	47782	11.401	**<0.001**	ANOVA
	Water × *Diet*	1	549	549	0.131	0.718	(rank trans-
	Residuals	224	938760	4191			formed)
Total FA content	*Water*	1	266	266.3	0.578	0.452	Two-way
	Diet	1	1271	1271.2	2.759	0.105	ANOVA
	Water × *Diet*	1	88	87.8	0.191	0.665	
	Residuals	36	16587	460.8			
SAFA content	*Water*	1	51.2	51.22	0.953	0.336	Two-way
	Diet	1	220.7	220.72	4.104	0.050	ANOVA
	Water × *Diet*	1	9.3	9.30	0.173	0.680	
	Residuals	36	1936.0	53.78			
MUFA content	*Water*	1	39	38.71	0.382	0.541	Two-way
	Diet	1	166	166.23	1.640	0.209	ANOVA
	Water × *Diet*	1	33	33.48	0.330	0.569	
	Residuals	36	3650	101.38			
PUFA content	*Water*	1	8.6	8.64	0.386	0.539	Two-way
	Diet	1	62.5	62.47	2.789	0.104	ANOVA
	Water × *Diet*	1	0.3	0.29	0.013	0.910	
	Residuals	36	806.4	22.40			
NLFA compo-	*Water*	1	0.00783	0.01709			PERMANOVA
sition of	*Diet*	1	0.03068	0.06695	0.690	0.475	(square root
gammarids	*Water* × *Diet*	1	0.01081	0.02359	2.701	0.099	transformed)
	Residuals	36	0.40896	0.89236	0.952	0.344	

Table S7 Number of analyzed replicates and group medians (with ± 95 % CI) of microbial parameters describing leaf quality used during the 24-day long-term assay. Moreover, the statistical test used for the respective response variable as well as the *p*-value from the statistical comparison of 15 + 500 µg/L AZO+CIP with the control is shown.

Endpoint	AZO + CIP in µg/L	*n*	Median	±95 % CI	Statistical test	*p*-value
Fungal biomass in µg ergosterol/g leaf dry mass	0 + 0	12	42.59	25.60 to 59.68	Wilcoxon rank-sum	0.478
	15 + 500	12	49.07	28.89 to 100.00		
Bacterial density in 10^9 cells/g leaf dry mass	0 + 0	12	0.35	0.23 to 0.64	Student's *t* (unpaired)	0.405
	15 + 500	12	0.30	0.18 to 0.52		
Fungal spores/mg leaf dry mass	0 + 0	12	35.75	12.56 to 102.17	Wilcoxon rank-sum	0.932
	15 + 500	12	34.28	19.90 to 53.38		
Sporulating fungal species	0 + 0	12	4.00	3.00 to 5.00	Student's *t* (unpaired)	0.839
	15 + 500	12	3.50	3.00 to 4.00		
Hyphomycete community composition	0 + 0	12	-	-	PERMANOVA	0.187
	15 + 500	12	-	-		
Total FA content in µg/mg leaf dry mass	0 + 0	11	16.80	13.04 to 24.97	Student's *t* (unpaired)	0.703
	15 + 500	11	17.91	12.35 to 22.53		
Sat. FA content in µg/mg leaf dry mass	0 + 0	11	6.74	5.33 to 9.24	Student's *t* (unpaired)	0.903
	15 + 500	11	7.26	5.04 to 9.04		
Monounsat. FA content in µg/mg leaf dry mass	0 + 0	11	1.43	1.36 to 1.92	Student's *t* (unpaired)	0.181
	15 + 500	11	1.45	1.05 to 1.75		
Polyunsat. FA content in µg/mg leaf dry mass	0 + 0	11	8.80	5.79 to 13.34	Student's *t* (unpaired)	0.602
	15 + 500	11	9.20	5.39 to 11.96		
FA composition of leaves	0 + 0	11	-	-	PERMANOVA	0.216
	15 + 500	11	-	-		

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Appendix A4

Herbicide-induced shifts in the periphyton community composition indirectly affect feeding activity and physiology of the gastropod grazer *Physella acuta*

Konschak, M., Zubrod, J.P., Duque Acosta, T.S., Bouchez, A., Kroll, A., Feckler, A., Röder, N., Baudy, P., Schulz, R., Bundschuh, M.

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Abstract

Herbicides are well known for unintended effects on freshwater periphyton communities. Large knowledge gaps, however, exist regarding indirect herbicide impacts on primary consumers through changes in the quality of periphyton as food source (i.e., diet-related effects). To address this gap, the grazer *Physella acuta* (Gastropoda) was fed for 21 days with periphyton that grew for 15 days in the presence or absence of the herbicide diuron (8 µg/L) to quantify changes in the feeding rate, growth rate and energy storage (neutral lipid fatty acids; NLFAs) of *P. acuta*. Periphyton biomass, cell viability, community structure and FAs served as proxies for food quality that support a mechanistic interpretation of the grazers' responses. Diuron changed the algae periphyton community and fatty acid profiles, indicating alterations in the food quality, which could explain differences in the snails' feeding rate compared to the control. While the snails' growth rate was, despite an effect size of 55 %, not statistically significantly changed, NLFA profiles of *P. acuta* were altered. These results indicate that herbicides can change the food quality of periphyton by shifts in the algae composition, which may affect the physiology of grazers.

Keywords

Biofilms, Bottom-up effects, Diatoms, Diuron, Fatty acids

Introduction

Freshwater periphyton, defined as submerged substrate-associated biofilm composed primarily of algae (especially diatoms and chlorophytes) and bacteria, but also harboring other organisms (fungi, protozoans and metazoans), play a pivotal role in the aquatic nutrient cycling (Wu, 2017). More specifically, periphyton is crucial for stream food webs as high-quality food source for herbivorous macroinvertebrates (i.e., grazers), providing essential macronutrients such as highly unsaturated fatty acids (HUFAs; Torres-Ruiz et al., 2007; Guo et al., 2018). HUFAs are critical for the somatic growth, development and reproduction of aquatic consumers (Brett and Müller-Navarra, 1997). Especially diatoms are rich in HUFAs (e.g., 20:5n-3), while chlorophytes and cyanobacteria are generally poor in these FAs (Brett et al., 2012). Therefore, shifts in the periphyton community composition induced by external factors (e.g., nutrients and chemical stressors) can alter the abundance of essential macronutrients, such as HUFAs, and thus change the food quality for grazers and

consequently their activity (e.g., feeding activity) and physiology (Guo et al., 2016; Crenier et al., 2019).

Among those external factors, herbicides, which are one major pesticide group detected in European rivers (Schreiner et al., 2016), pose a potential hazard for periphyton-grazer systems. Due to their modes of action that target vital processes in non-target algae (Nagai et al., 2016), herbicides affect periphyton communities (Schmitt-Jansen and Altenburger, 2005a; McClellan et al., 2008) and ultimately impact the ecosystem functions they provide (Krieger et al., 1988; Brust et al., 2001). In this context, studies suggest that herbicides can indirectly affect the physiology and behavior of grazers by altering the food quantity and quality of periphyton (i.e., dietary pathway; Rosés et al., 1999; Rybicki et al., 2012; Rybicki and Jungmann, 2018). At the same time, herbicides are considered less toxic to macroinvertebrates via waterborne exposure (Brock et al., 2004). However, the test designs of published studies directly applied herbicides via the water phase as well as did not exclude interactive effects of waterborne herbicide exposure and potential biotic stress (i.e., through intraspecific interactions between snails) obscuring indirect effects on the individual level. Moreover, food quality parameters, such FAs profiles, were not quantified during these studies to link shifts in periphyton communities with altered food quality.

The aim of the present study was, therefore, to assess diet-related effects on the grazers' feeding activity and physiology (i.e., growth and energy storage via neutral lipid fatty acids; NLFAs) by excluding intraspecific interactions and direct herbicide application into the water phase. Individuals of the model-grazer *Physella acuta* (Gastropoda), a cosmopolitan freshwater scraper-grazer (Vinarski, 2017), were fed for 21 days with periphyton, which grew in the absence or presence of the herbicide diuron. The photosystem II inhibitor diuron was selected as model herbicide because sufficient literature is available facilitating the interpretation of potential effects to be observed in the present study. Based on published data, we applied 8 µg diuron/L, a concentration that did not induce waterborne effects in *P. acuta* (López-Doval et al., 2014) but showed clear effects on periphyton communities (Pesce et al., 2006; Ricart et al., 2009; Magnusson et al., 2012). To reveal the mechanisms that cause diuron-induced changes in the food quality of periphyton for *P. acuta*, its biomass, community structure and FA profiles as proxy for food quality (in particular HUFAs) were characterized.

We hypothesized that diuron exerts a selection pressure on periphyton during its development leading to a shift in the community composition (Ricart et al., 2009; Magnusson

et al., 2012; Sgier et al., 2018). These potential alterations in community composition, in turn, would change the nutritional quality of periphyton for the gastropod grazer and could be reflected in changes in its feeding activity and physiology; with similar effects being observed for fungicides affecting heterotrophic processes (cf. Zubrod et al., 2015).

Materials and methods

Diuron effects on periphyton

Source of periphyton

One day prior to the initiation of the colonization of ceramic tiles by periphyton, five stones of similar size and periphyton coverage were gathered from the stream Eußerbach (49°15´N; 7°57´E). The stream is located within a nature reserve and upstream of intensive agricultural land use and effluent discharges. In the laboratory, periphyton of the sun-exposed surface of each stone was scraped off with a cell scraper (VWR®, Radnor, US-PA). The scraped periphyton was suspended in 1.5 L of an algae medium (i.e., KUHL by Kuhl and Lorenzen, 1964; Table S1) and kept overnight in darkness at 20 ± 1°C under continuous stirring (200 rpm) and aeration.

Periphyton colonization and diuron application

Periphyton colonization on ceramic tiles in absence or presence of 8 µg diuron/L (i.e., control and diuron treatment) was performed at the Landau Laboratory Stream Microcosm Facility. Periphyton on these tiles served as food for *P. acuta* over the study duration of 21 days. To ensure the provisioning of food at a constant quantity (i.e., *ad libitum* feeding) over the entire study duration, three independent colonization phases were performed that guaranteed a supply of fresh periphyton every 7 days (see Fig. 1). Each colonization phase consisted of three stainless-steel channels (120 cm × 30 cm × 20 cm) per treatment (in total 18 channels; Fig. 1a) and lasted for 21 days (i.e., 6-day diuron-free colonization phase followed by a 15-day exposure period). Channels were pseudo-randomly positioned in a cooling water bath (Lauda WK 450; Lauda Dr. R. Wobser GmbH & Co. KG, Lauda-Königshofen, Germany), to minimize location effects during microbial colonization (Fig. 1a). Each channel contained a stainless-steel paddle wheel facilitating a velocity of 0.12 ± 0.01 m/s (measured in the area of tiles). Moreover, 60 burned (for 5 hours at 500 °C) glazed ceramic tiles (4.7 cm × 4.7 cm) serving as periphyton substrate and 30 L of KUHL medium (Fig. 1b) were added. The facility was equipped with 400 W sodium-vapor and metal halide lamps (SON-T Agro and Master

HPI-T Plus, Koninklijke Philips N.V., Amsterdam, Netherlands) providing photosynthetic light. Photon flux density (180.83 ± 8.74 µmol/m²s) and water temperature during a 16:8 hour light:dark photoperiod (15.48 ± 0.64 during night and 20.17 ± 0.65°C during the day) were recorded throughout the study (Figure S1 and Table S2).

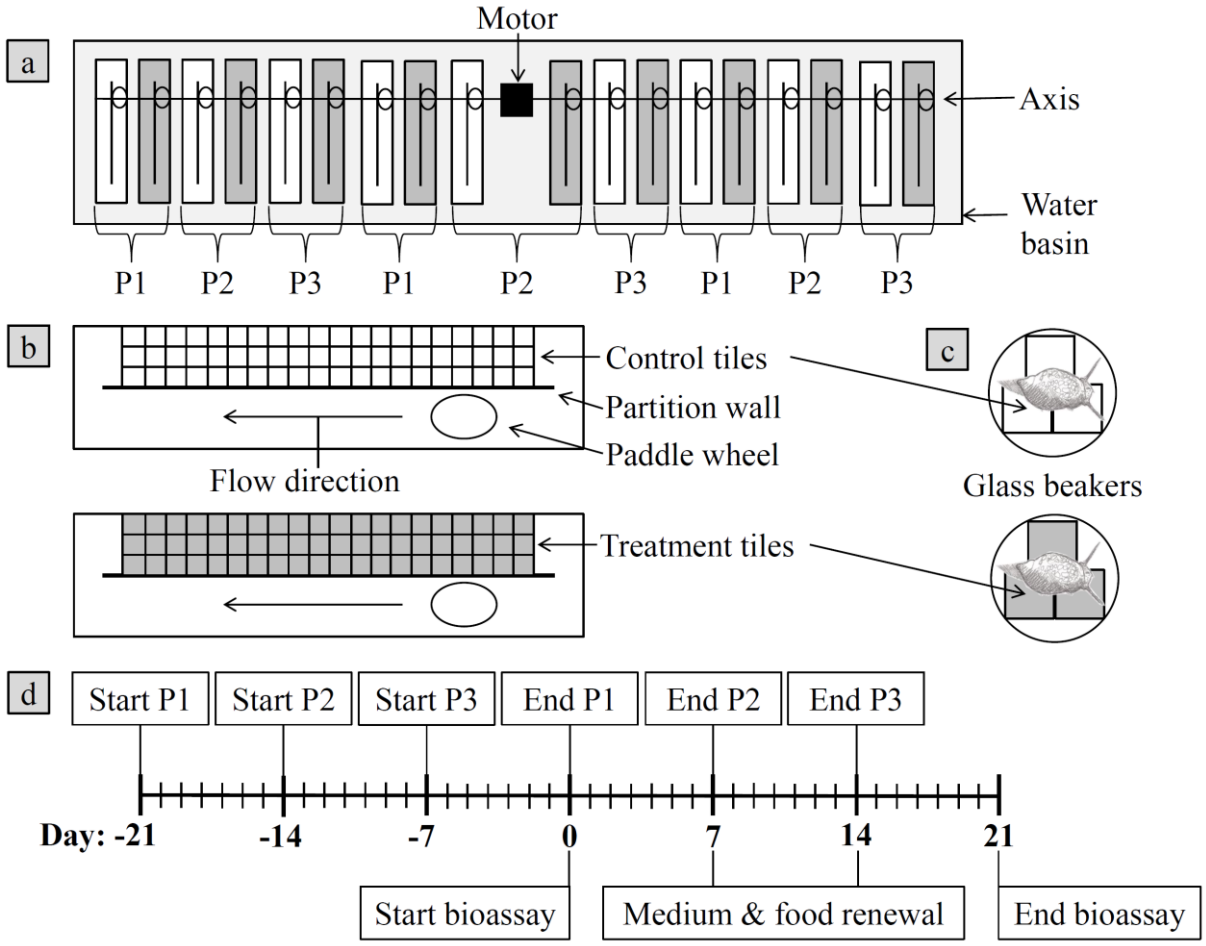

Fig. 1 (**a**) Schematic overview of the indoor stream microcosm facility where white and grey channels represent the control and the diuron treatment, respectively, and P1 – 3 are the three independent periphyton colonization phases partly running in parallel. (**b**) illustrates the setup of the experimental stream channels and (**c**) schematizes the replicates of the bioassay with *P. acuta*. (**d**) constitutes the timeline of events in the microbial colonization and the 21-day feeding bioassay. See text for details.

At the start of each colonization phase (the first started on November 18th 2018), 250 mL of the cell suspension of scraped periphyton was added to each channel. To allow an adaptation of the periphyton community to the KUHL medium as well as laboratory conditions and to ensure an initial colonization (cf. Ricart et al., 2009), the first medium renewal and diuron application were performed 6 days after the colonization was initiated. Therefore, 25 L of the KUHL medium with the respective diuron concentration (5 L of the old

medium was left in the channel to protect the periphyton from drying out) was renewed every third day until the end of the colonization phase. To achieve the nominal concentration of 8 µg/L, the commercially available product Diuron 80 (Schneiter AGRO AG, Seon, Switzerland) was diluted in 30-L stainless-steel containers filled with KUHL medium and 25 L of it was transferred in each treatment channel. However, at day 6, a nominal concentration of 9.6 µg/L was applied to achieve 8 µg diuron/L in the channels as the spiked medium had to be mixed with 5 L of uncontaminated KUHL medium. Before each medium renewal, pH, dissolved oxygen, electrical conductivity and flow velocity were measured (Table S2). At the end of each colonization phase, tiles, which were fully and uniformly covered with periphyton, were either randomly introduced into the microcosms of the feeding bioassay (see *21-day feeding bioassay*) or preserved for microbial or FA analyses.

To verify diuron concentrations, samples of each channel were taken immediately before and after the medium renewal and stored at −20 °C. Concentrations were measured via ultra-high-performance liquid chromatography-mass spectrometry (Thermo Fischer Inc., Waltham, US-MA; cf. Zubrod et al., 2015) and matrix-aligned external standards (Diuron PESTANAL®, Sigma-Aldrich, St. Louis, US-MO). As mean measured diuron concentrations deviated by +16 % in fresh and −10 % in 3-day old medium (Table S3), the nominal concentration of 8 µg/L is reported throughout the entire study.

Analyses of microbial parameters

To shed light into the underlying mechanisms of potential diet-related diuron effects on *P. acuta*, the periphyton was characterized through assessments of ash-free dry mass (AFDM), photosynthetic pigments, cell viability as well as the composition of phenotypes (i.e., size and shape of cells) and diatoms as measures for the algae community structure. Therefore, eight tiles per channel were directly taken from the stream microcosms at termination of each colonization phase. Periphyton surface was measured by taking digital images (Apple iPhone 6, Cupertino, US-CA) to assess the coverage via ImageJ version 1.52a (Schneider et al., 2012). Afterwards, periphyton was removed with a cell scraper and suspended in 250 mL SAM5-S medium (Borgmann, 1996). For AFDM analysis, an aliquot of 10 mL of the cell suspension was preserved at −20 °C (n = 3 channels × 3 phases = 9). To determine photosynthetic pigments (i.e., chlorophyll *a*, *b* and $c_1 + c_2$), 10 mL were filtered through a burned glass fiber filter (GF 6, Whatman®, Maidstone, United Kingdom) and stored at −20 °C (n = 9). The remaining cell suspension was filled in 50-mL centrifuge tubes and preserved at −20 °C for analyzes of the microbial community (n = 9) as well as FAs (n = 9).

Periphyton biomass

To determine the total organic content per sample (n = 9 per control and treatment) in form of AFDM, samples were defrosted and filtered through pre-weighed GF 6 glass fiber filters. Filters were subsequently dried for 24 hours at 60 °C, weighed to the nearest 0.01 mg, burned for 5 hours at 500 °C and re-weighed (cf. Biggs and Kilroy, 2000). AFDM was calculated as per Biggs and Kilroy (2000), extrapolated to the total sample and normalized to the photographed surface area of the periphyton (i.e., g/m^2).

Chlorophyll *a* (Chl *a*), *b* (Chl *b*) and $c_1 + c_2$ (Chl *c*) were quantified via spectrophotometry (n = 9 per control and treatment), as indicator for the total mass of autotrophic organisms (Biggs and Kilroy, 2000), chlorophytes and diatoms, respectively (Hagerthey et al., 2006). Biomass for the latter taxonomic groups was determined, since freshwater periphyton mainly consists of chlorophytes and diatoms (Wu, 2017). Following Feckler et al. (2018), Chls were extracted in 10 mL 90 % acetone by 2 min of ultrasonication. Samples were centrifuged for 15 min at 3,500 rpm and 300 µL of each supernatant was subsequently transferred into an individual well of a transparent polystyrene microwell plate (Nunc™ F96 MicroWell™, Thermo Fisher Scientific Inc., Waltham, US-MA). The absorbance of pigment extracts was measured at the wavelength spectrum of 450 – 750 nm by using a microplate plate reader (Infinite® 200 PRO, Tecan Group AG, Männedorf, Switzerland). Chl *a*, *b* and *c* were calculated as per Jeffrey and Humphrey (1975), extrapolated to the total sample and normalized to the surface area (i.e., mg/m^2; see above).

Phenotype-based community composition and cell viability

To characterize shifts in the algae community composition and cell viability (i.e., proportion of living and dead cells; 9 samples per control and treatment), three technical replicates of each filtered sample (conserved in a 0.01 % paraformaldehyde/0.1 % glutaraldehyde solution and stored at −20 °C prior to transport) were measured with a Beckman Coulter Gallios flow cytometer equipped with three lasers (405, 488 and 638 nm) for 10 fluorescence parameters from 450 – 755 nm as well as forward and side scatter (in total 12 parameters). The protocol is optimized for phototrophic cells but also detects inorganic particles and is described in detail in Sgier et al. (2016). For each sample, 10,000 events were obtained and gated to select the events that were within the signal saturation limit of the 12 parameters (> 99 %). The area signal intensity per parameter was converted into a CSV file and visualized with viSNE via the cyt software. The latter creates a two-dimensional scatter plot in which phenotypically

similar cells of different samples are assigned to groups (i.e., visually separable clusters, also called viSNE clusters) based on optical properties (scattered light, fluorescence). The number of cells in each viSNE cluster can then be quantified for each sample.

Diatom community composition

Since periphytic communities are often dominated by diatoms (Wu, 2017) that constitute the most nutritious taxonomic group for aquatic animals (Brett and Müller-Navarra, 1997), diatom communities were structurally analyzed (9 samples per control and treatment) using DNA metabarcoding and high-throughput sequencing (Mortágua et al., 2019). Briefly, DNA of each sample was extracted and the 312 bp region of the *rbcL* gene from three technical replicates per sample was amplified with PCR using forward (Diat_rbcL_708F) and reverse (R3) primers (Vasselon et al., 2017). For each sample, PCR amplification was performed in triplicates with 25 ng of DNA in a 25-µl PCR reaction conducted under the following conditions: 95°C for 15 min, 30 cycles of 45 s at 95°C, 55°C for 45 s, and 72°C for 45 s. PCR products were then quantified using a fluorometer (Qubit®, Invitrogen, Thermo Fisher Inc., Waltham, US-MA) and sent to the Genome Transcriptome platform (Bordeaux, France) for Illumina MiSeq analysis with the V2 paired-end sequencing kit (2 × 250 bp).

Raw data (with barcode- and adapter sequences removed) were processed with R JAMP (https://github.com/VascoElbrecht/JAMP, last accessed on 20/04/2020). Paired-end merging was performed by usearch (v8.1.1756; Edgar, 2013). Subsequently, primer sequences were removed via cutadapt (version 2.6; Martin, 2011). A quality-check discarded sequences outside the target length of 264 ± 10 bp and with more than 1 expected error. Resulting DNA reads were combined into operational taxonomic units (OTUs) by using a distance similarity threshold of 2 %. The removal of chimeric sequences was conducted to eliminate the sequencing artifacts. All sequences (including singletons) were matched against the OTUs in usearch. The obtained OTUs were taxonomically assigned using the curated open-access diatom database (Diat.barcode; Rimet et al., 2019). Gene copy numbers of diatom species were corrected to their individual biovolume (see Vasselon et al., 2018 for more details).

Fatty acid analysis of periphyton

To characterize periphyton food quality for grazers, total FAs were quantified (9 samples per control and treatment). Furthermore, FAs also aid the assessment of the basal (i.e., higher taxonomic groups) periphyton community structure (Honeyfield and Maloney, 2015). Lyophilized periphyton samples were weighed to the nearest 0.01 mg. Total FAs of

periphyton were derivatized to fatty acid methyl esters (FAMEs) following Fink (2013), since this method increased the purification of the analyzed samples in comparison to the rapid transesterification with trimethylsulfonium hydroxide (TMSH) used for NLFA analysis of snails (see *Neutral lipid fatty acid analysis of* P. acuta). Briefly, lipids were extracted in 5 mL of a chloroform/methanol mixture (2:1). FAs were transesterified into FAMEs via 3N methanolic HCl (Sigma-Aldrich, St. Louis, US-MO) and the latter were liquid-liquid extracted by using isohexane. FAMEs were analyzed via gas chromatography with flame-ionization detection (CP-3800, Varian, Palo Alto, US-CA) using a DB-225 GC column (30 m, ID 0.25 mm, film thickness 0.25 µm, J&W Scientific, Agilent Technologies, Santa Clara, US-CA; cf. Fink, 2013) and nitrogen as carrier gas. FAMEs were detected via retention times of FAME standards (Sigma-Aldrich, St. Louis, US-MO) and FA concentrations in µg FA/mL were quantified using external standard calibration. The FA concentration of each sample was adjusted by the recovery rate of the internal standard as well as via blank correction. Finally, concentrations were extrapolated to the total volume and normalized to the dry weight of the sample (i.e., µg FA/mg periphyton dry mass).

Diet-related diuron effects on *P. acuta*

Source of snails

Two weeks before the start of the feeding bioassay, 100 *P. acuta* with a shell length of 6 – 8 mm were taken from our in-house culture. Snails were kept at 20 ± 1°C in a separate 8-L aquarium filled with the culture medium SAM-5S under continuous aeration and a 16:8 hour light:dark photoperiod (990 ± 100 lx provided by fluorescence tubes; Osram L 58W/965 Biolux, Osram GmbH, Munich, Germany). Animals were fed *ad libitum* with dried stinging nettles (*Urtica* spp.) to align their diet.

21-day feeding bioassay

After termination of each periphyton colonization, one tile per channel ($n = 3$; separated by treatment), was transferred into one replicate (microcosm) of the 21-day feeding bioassay with *P. acuta*. Each microcosm of the control and the treatment (i.e., periphyton exposed to 0 or 8 µg diuron/L; $n = 30$; Fig. 1c) of the feeding bioassay consisted of a 2-L glass beaker filled with 0.5 L of aerated SAM-5S, one snail, and three tiles colonized by periphyton. The bioassay was performed at 20.5 ± 0.2°C, under continuous aeration (8.7 ± 0.1 mg O_2/L) and a 16:8 hour light:dark photoperiod (985.4 ± 37.9 lx provided by Biolux fluorescence tubes). Every 7 days, medium and food were renewed by transferring snails into a microcosm

containing three tiles covered with fresh periphyton. The periphyton remains on the tiles of the previous microcosm were removed from the tiles with a cell scraper and preserved in 50-mL centrifuge tubes (SuperClear™, VWR®, Radnor, US-PA) at −20 °C to determine its AFDM. At the end of the bioassay, water adhering to snails was gently removed with a tissue, and individuals were immediately shock-frozen in liquid nitrogen and stored at −80 °C for NLFA analysis. Snails that died during the bioassay (5 per control and treatment) were excluded from further analysis.

The feeding rate as proxy for the feeding activity of *P. acuta* was estimated via the surface area and AFDM of periphyton on ceramic tiles. Therefore, the periphyton surface area before and after the 7-day feeding period was measured by taking digital images (Apple iPhone 6, Cupertino, US-CA) and assessing coverage using ImageJ version 1.52a (Schneider et al., 2012). To quantify AFDM, the periphyton after the 7-day feeding period was defrosted and, due to the high periphyton biomass, diluted in 150 mL SAM5-S. A 15-mL aliquot of each sample was filtered through a pre-weighed GF 6 glass fiber filter. Filters were dried, weighed, burned, re-weighed and AFDM was calculated as described in *Periphyton biomass*.

Snails' feeding rate (*F*) was expressed in mg/day and estimated as follows:

$$F = \frac{AFDM \times k - AFDM}{t}$$

where *AFDM* is the ash-free dry mass of periphyton in mg after 7 days of feeding, *k* is the quotient of the surface of the fresh periphyton divided by the surface of the same periphyton after 7 days of feeding and *t* is the observation time in days. Growth of *P. acuta* in µg dry mass/day was estimated by subtracting the mean dry mass of 23 lyophilized snails at the start of the 21-day feeding bioassay from the final dry mass of each individual divided by the study duration in days.

Neutral lipid fatty acid analysis of P. acuta

NLFAs of snails were determined ($n = 6$ per control and treatment), since they represent the most important energy storage in invertebrates (Azeez et al., 2014). Moreover, alterations in the diet can change NLFA composition (in comparison to phospholipid FAs) relatively fast (Iverson, 2012). Snails were lyophilized and weighed to the nearest 0.01 mg. Three *P. acuta* of the same treatment were pooled and manually crushed. Afterwards, 5 mL chloroform and the internal standard (Tristearin-D105, Larodan, Solna, Sweden) was added and the samples

were stored over night at 4°C. As described in Konschak et al. (2020), neutral lipids were extracted via solid-phase extraction (Chromabond® easy polypropylene columns, Macherey-Nagel, Düren, Germany) and derivatized into FAMEs using TMSH (Sigma-Aldrich, St. Louis, US-MO). FAMES were determined and NLFAs in µg/mg snail dry mass were calculated as described in *Fatty acid analysis of periphyton*.

Statistical analyses

Statistical tests with additional information (e.g., *p*-values, F-statistics, sum and mean of squares, group medians with 95 % confidence intervals) are provided in Tables 1 and S4–S8. Briefly, data normality and homogeneity of variances were tested via Shapiro–Wilk and Levene's test as well as by visually inspecting data and model residuals, respectively. Two-level hierarchical data (i.e., repeated measurements of the feeding rate are nested within the snails) were analyzed using multilevel modeling. The full model comprised the continuous predictor variable *Time*, the factorial predictor variable *Diuron* (i.e., fed with periphyton colonized in the absence or presence of diuron), the interaction of both (*Time* × *Diuron*) and random intercepts (i.e., intercepts varied across snails). For testing statistical significance of each predictor variable on the response variable, the likelihood ratio test was applied (for details, see Table S4; Field et al., 2012). Prior to applying the models, right-skewed data were log-transformed (Table S4). Multilevel modeling was also performed for the grazed surface area of periphyton showing that the estimation of the feeding rate does not distort the statistical outcome (see Fig. S5 and Table S4 for more details). Parametric and non-parametric data with one factorial predictor with two levels were analyzed via Student's *t*-test and Wilcoxon rank-sum test, respectively (AFDM, Chls, cell viability, phenotype-based cell groups, FAs as well as feeding rate per week, growth rate and NLFAs of *P. acuta*; Table 1, S5, S7, S8). Multivariate data were evaluated by using permutational multivariate analysis of variance (PERMANOVA) with Bray–Curtis distances (composition of phenotype-based cell groups, diatoms and (NL)FAs; Table 1). Prior to testing via PERMANOVA, data were square-root transformed to reduce the discriminatory power of dominant groups and multivariate homogeneity of group dispersion was checked avoiding statistically significant differences from within-group variability (i.e., dispersion effect). Non-metric multidimensional scaling (NMDS) with Bray-Curtis distances was applied to visualize the (dis-)similarities of the phenotype-based cell group and (NL)FA composition of the control and diuron treatment. Diversity of OTUs was estimated via the Simpson and Shannon index. Statistical analyses were carried out and figures were prepared with R Version 4.1.0 for

Windows (R Core Team, 2014) and the add-on packages *asbio*, *car*, *ggplot2*, *mvoutlier*, *nlme*, *plotrix* and *vegan*.

Please note that the term "significant" describes statistical significance ($p \leq 0.05$) throughout the results and discussion section. Moreover, the present study takes criticisms on null hypothesis significance testing into account. Instead of basing the interpretation of data only on the arbitrary defined alpha threshold of 0.05, we also make use of effect sizes and their 95 % confidence intervals as measure for the magnitude and relative importance of an effect (Nuzzo, 2014).

Results and discussion

Diuron effects on periphyton

Diuron did not significantly affect viability of algae or total periphyton biomass (i.e., AFDM and Chl contents), despite a trend towards increased Chl *a*, *b* and *c* levels (Table 1). However, cell cytometry results showed a significant shift in phenotypes (i.e., size and shape) and fluorescence intensities in periphyton exposed to diuron (Fig. 2; Table 1). Groups with large cells (G 01, G 02, G 04, G 08; high forward scatter) were those that remained stable or significantly increased (↑) in relative abundance, whereas the relative abundance of groups with smaller cells (G 03, G 07, G 12, G 14; low forward scatter) significantly decreased (↓) or remained stable (Fig. 2, Table S5). Likewise, groups with stronger fluorescence, which also constitutes the groups with large cells (Table S2), increased or remained stable, while those with lower fluorescence decreased or remained stable. In a previous experiment on periphyton exposed to 20 µg/L diuron by Sgier et al. (2018), higher relative abundance of red-algae like cells, identified as *Bangia* sp., was detected. In the same study, the proportion of decaying cells was higher. However, an increase in red-algae like cells and decaying cells could not be identified in the present study. Irrespective of that, observed phenotype changes could be shifts in the community composition, due to interspecific variability in species sensitivity to herbicide stress, and physiological adaptations within algae species (e.g., increase of Chls) based on a selection pressure by diuron, favoring specific phenotypes (Schmitt-Jansen and Altenburger, 2005b).

Table 1 Medians (with 95 % CIs) of periphyton- and snail-related endpoints. Moreover, applied statistical tests as well as *p*-values from statistical comparisons of the diuron treatment with the control are shown. All *p*-values below 0.05 are printed in bold.

Organism(s)	Endpoint	*Predictor*/ Treatment	Median	±95 % CI	Statistical test	*p*-value
Periphyton	AFDM in g/m^2	Control	19.97	10.94 to 24.78	Student's *t*	
		Diuron	18.65	15.77 to 22.05		0.803
	Chl *a* in mg/m^2	Control	218.59	88.52 to 307.19	Student's *t*	
		Diuron	305.51	205.13 to 336.31		0.112
	Chl *b* in mg/m^2	Control	51.22	51.21 to 66.45	Student's *t*	
		Diuron	74.82	50.75 to 135.98		0.054
	Chl *c* in mg/m^2	Control	24.17	14.05 to 36.62	Student's *t*	
		Diuron	34.68	24.09 to 49.70		0.108
	Living cells in %	Control	35.30	32.75 to 36.52	Wilcoxon rank-sum	
		Diuron	37.43	33.15 to 61.20		0.297
	Phenotype-based cell groups	*Phase*	-	-	PERMANOVA	0.295
		Diuron	-	-		**0.004**
		Phase × *Diuron*	-	-		0.254
	Diatom community composition	*Phase*	-	-	PERMANOVA	0.154
		*Diuron**	-	-		0.071
		Phase × *Diuron*	-	-		0.667
	Rel. abundance of *Nitzschia palea* in %	Control	90.67	81.73 to 93.26	Wilcoxon rank-sum	
		Diuron	70.32	43.96 to 88.98		**0.019**
	Rel. abundance of all other diatoms in %	Control	9.33	6.74 to 18.26	Wilcoxon rank-sum	
		Diuron	29.68	11.02 to 56.04		**0.019**
	FA composition (absolute)	*Phase**	-	-	PERMANOVA	0.067
		Diuron	-	-		**0.002**
		Phase × *Diuron*	-	-		0.370
	FA composition (relative)	*Phase*	-	-	PERMANOVA	**0.008**
		Diuron	-	-		**0.005**
		Phase × *Diuron*	-	-		0.891
Snails	Feeding rate in mg/day	-	-	-	Likelihood ratio	
		Time	-	-		0.161
		Time + *Diuron*	-	-		0.528
		Time × *Diuron*	-	-		**<0.001**
	Feeding rate in mg/day (7. day)	Control	0.84	0.31 to 2.42	Wilcoxon rank-sum	
		Diuron	0.39	0.22 to 0.91		0.147
	Feeding rate in mg/day (14. day)	Control	0.71	0.12 to 1.19	Wilcoxon rank-sum	
		Diuron	0.98	0.46 to 1.42		0.186
	Feeding rate in mg/day (21. day)	Control	0.67	0.50 to 0.87	Wilcoxon rank-sum	
		Diuron	1.35	0.88 to 2.46		**0.003**
	Growth in µg/day	Control	51.68	18.82 to 105.49	Wilcoxon rank-sum	
		Diuron	80.72	43.11 to 122.15		0.299
	FA composition of grazers (absolute)	*Diuron*	-	-	PERMANOVA	**0.004**
	FA composition of grazers (relative)	*Diuron*	-	-	PERMANOVA	**0.004**

*Heterogeneous multivariate dispersions

Herbicide-induced changes in the algae community composition are supported by shifts in relative sequence abundances of diatoms in the presence of diuron (Fig. 3a, b), though the observed trend of the PERMANOVA analysis may be attributed to a dispersion effect (Table 1). The most dominant diatom species, most likely a diuron-sensitive cryptic species of *Nitzschia palea* (Esteves et al., 2017), was significantly negatively affected (Fig. 3a, Table 1). Consequently, the relative abundance of phenylurea herbicide tolerant diatom species, such as *Gomphonema parvulum* (Figure 3a; Table S6; Larras et al., 2014), was elevated pointing towards pollution-induced alterations in the community structure. Moreover, the exposure towards 8 µg diuron/L had a positive influence on the diversity of diatoms (Fig. 3c). The chemical stressor may have inhibited species dominating the community under present laboratory conditions and, due to a reduced competitive pressure, enabled diatom species with less favorable traits under the given conditions to propagate (Serra et al., 2009).

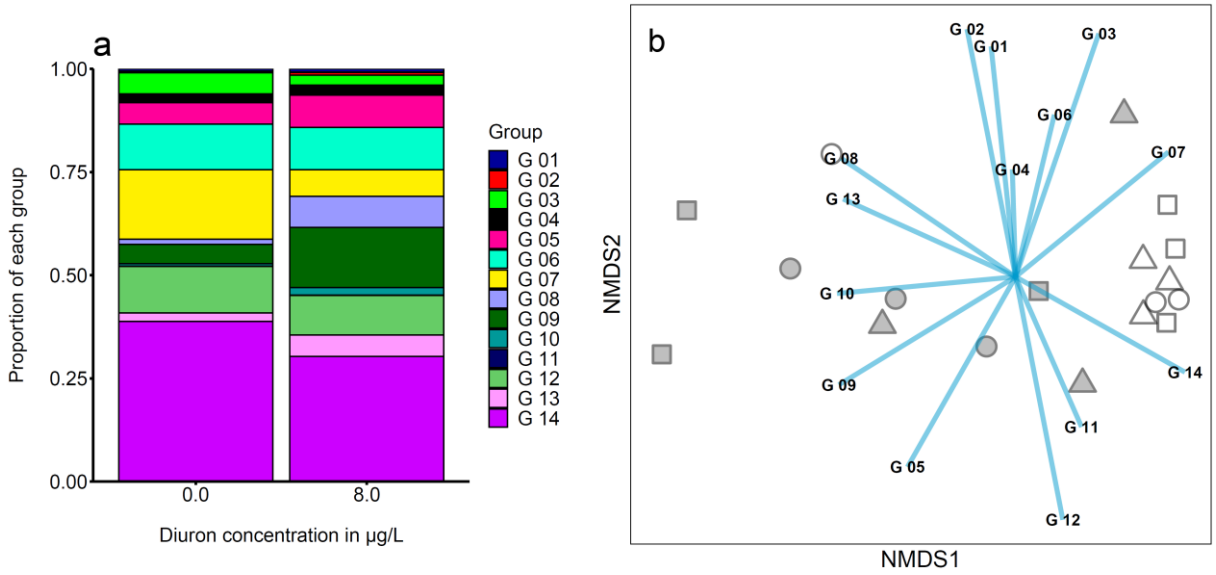

Fig. 2 (**a**) Mean relative abundance (0 – 1) and (**b**) Non-metric multidimensional scaling (NMDS) plots for phenotype-based cell groups (G 01 – G 14) exposed to 0 (i.e., control; white symbols; $n = 9$) and 8 µg diuron/L (grey symbols; $n = 9$) during the periphyton colonization. Circles, squares and triangles constitute the first, second and third periphyton colonization phase, respectively. The stress value (as a measure of "goodness-of-fit") was below 0.2, indicating a reasonable fit (Clarke, 1993), and blue lines display the impact of each group. Statistical analyses are displayed in Table 1 and S5.

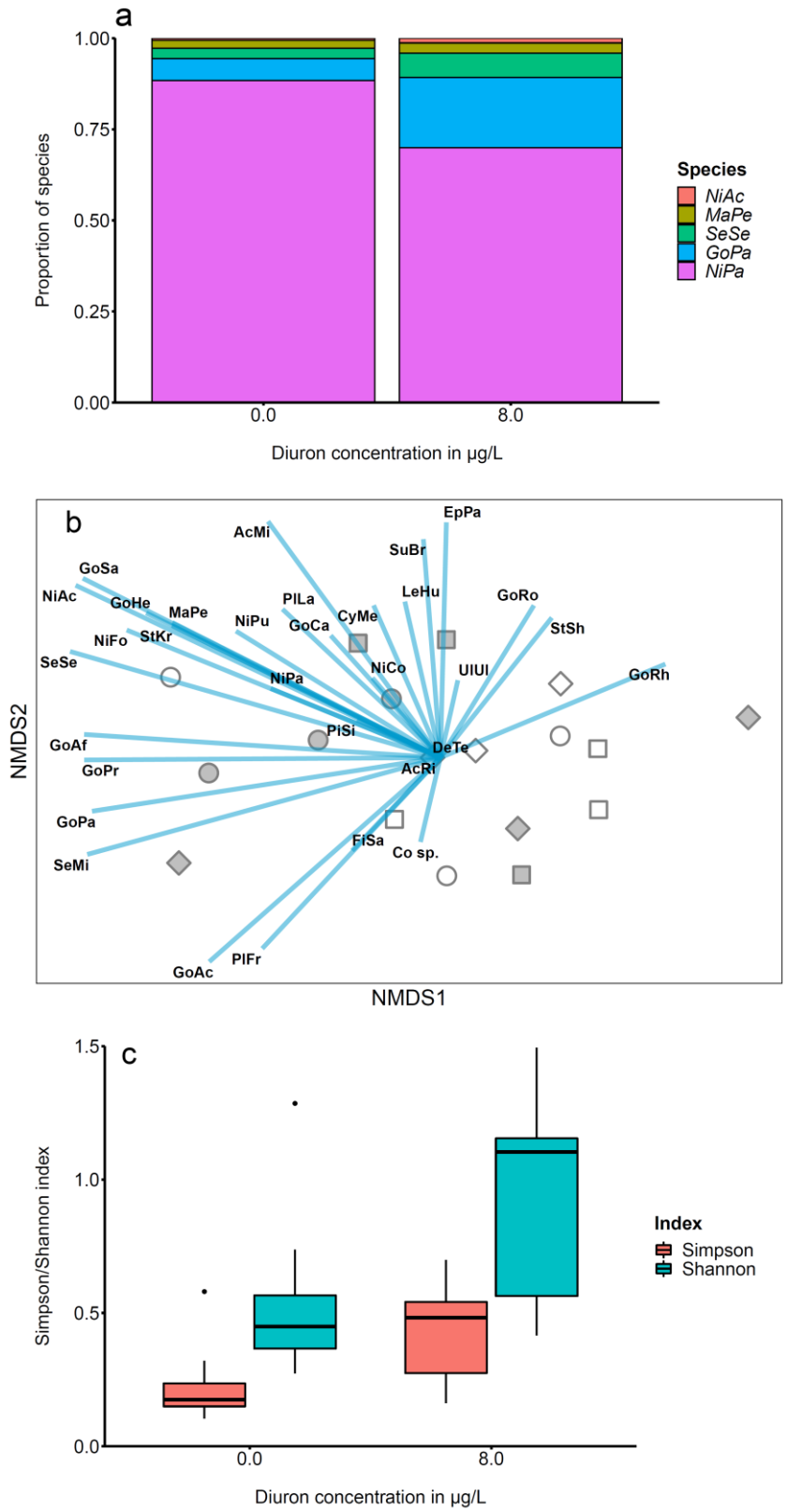

Fig. 3 (**a**) Mean relative sequence abundance (0 – 1) of the five most prevalent diatom species and (**b**) Non-metric multidimensional scaling (NMDS) plots for diatom composition of periphyton exposed to 0 (i.e., control, in white; $n = 9$) and 8 µg diuron/L (in grey; $n = 9$) during the periphyton colonization. Circles, squares and diamonds constitute the first, second and third periphyton colonization phase, respectively. The stress value (as a measure of "goodness-of-fit") was below 0.2, indicating a reasonable fit (Clarke, 1993), and blue lines

display the impact of each diatom species. Statistical analyses are displayed in Table 1. (**c**) Boxplots illustrating alpha diversity of diatoms (at the OTU level) in the absence and presence of 8 µg diuron/L by using Simpson and Shannon index. Abbreviations: AcMi (*Achnanthidium minutissimum*), AcRi (*Achnanthidium rivulare*), Co sp. (*Cocconeis* sp.) *Cyclotella meneghiniana* (CyMe), *Denticula tenuis* (DeTe), *Epithemia parallela* (EpPa), *Fistulifera saprophila* (FiSa), *Gomphonema acuminatum* (GoAc), *Gomphonema affine* (GoAf), *Gomphonema carolinense* (GoCa), *Gomphonema hebridense* (GoHe), *Gomphonema parvulum* (GoPa), *Gomphonema productum* (GoPr), *Gomphonema rhombicum* (GoRh), *Gomphonema rosenstockianum* (GoRo), *Gomphonema saprophilum* (GoSa), *Lemnicola hungarica* (LeHu), *Mayamaea permitis* (MaPe), *Nitzschia acidoclinata* (NiAc), *Nitzschia communis* (NiCo), *Nitzschia fonticola* (NiFo), *Nitzschia palea* (NiPa), *Nitzschia pusilla* (NiPu), *Pinnularia sinistra* (PiSi), *Planothidium frequentissimum* (PlFr), *Planothidium lanceolatum* (PlLa), *Sellaphora minima* (SeMi), *Sellaphora seminulum* (SeSe), *Stauroneis kriegeri* (StKr), *Staurosira shiloi* (StSh), *Surirella brebissonii* (SuBr), *Ulnaria ulna* (UlUl).

The conjectured shifts in the periphyton community composition induced by diuron should also be reflected in the FA profile, as FAs vary between different taxonomic groups (Taipale et al., 2013). The total amount of each FA significantly increased in the presence of 8 µg diuron/L or was comparable to the control (except for 22:0↓; Fig. 4a, Table S7). For relative FA abundances, 16:1n-7, 18:1n-7, 20:3n-3 and 20:4n-6 was significantly enhanced, while 22:0, 18:1n-7, 20:1n-9 and 18:3n-3 were significantly decreased (Table S7). The positive correlation between 16:1n-7 and HUFAs (e.g., 20:5n-3 and 20:4n-6) with periphyton colonized in the presence of diuron (Figure 4b) could be related to higher abundances of diatoms (Brett et al., 2012; Taipale et al., 2013), whereas the increase in 18:1n-7 (that also positively correlates with periphyton exposed to diuron) could indicate higher bacterial abundances (Torres-Ruiz et al., 2007; Torres-Ruiz and Wehr, 2010). The most abundant FA was 18:3n-3, a fatty acid characteristic for chlorophytes (Brett et al., 2012), in both the control and treatment. However, the relative abundance of this FA decreased in periphyton exposed to diuron indicating a reduction in chlorophytes. In this context, it is worth noting that chlorophytes seem to have dominated the periphyton community, while diatoms were barely present in periphyton, as indicated by low levels of 20:5n-3 (Table S7, S8) in the present study. This observation is unexpected, as diatoms are often the predominant taxonomic algae group in periphyton (Wu, 2017), which was also reflected in earlier studies (Molander and Blanck, 1992; McClellan et al., 2008; Tlili et al., 2008). This high abundance of chlorophytes was probably triggered by the environmental conditions during the experiment (i.e., nutrient-rich medium coupled with high light intensity; Hill et al., 2011; Cashman et al., 2013) and by the origin of the periphyton that may simply be dominated by chlorophytes. Irrespective of that, periphyton colonized in the presence of 8 µg diuron/L showed a higher total amount of

HUFAs (Table S7), a proxy for food of high quality for consumers (Brett and Müller-Navarra, 1997). Previous studies showed that high-quality food positively correlates with feeding and growth rates of invertebrates (Ahlgren et al., 1990; Guo et al., 2016; Vonk et al., 2016). Consequently, an increase in the feeding activity and somatic growth of *P. acuta* fed with diuron-exposed periphyton was expected.

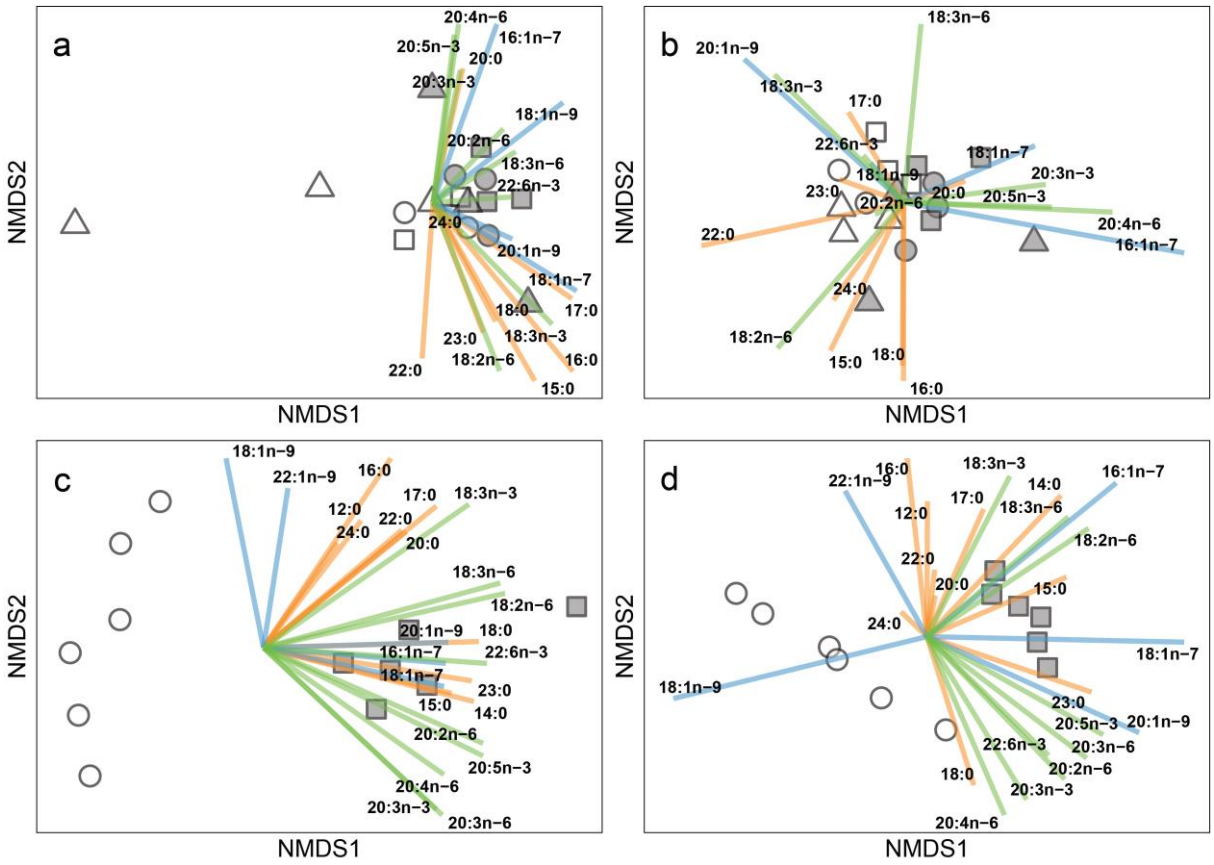

Fig. 4 Non-metric multidimensional scaling (NMDS) plots for the FA composition of periphyton exposed to 0 (i.e., control; white symbols; $n = 8$) and 8 µg diuron/L (grey symbols; $n = 9$) during the periphyton colonization in (**a**) total amounts (in µg/mg) and (**b**) relative abundances (0 – 1). Circles, squares and triangles constitute the first, second and third periphyton colonization phase, respectively. Moreover, the NLFA composition of snails fed with periphyton colonized under control conditions (white circles; $n = 6$) and in the presence of 8 µg diuron/L (grey squares; $n = 6$) during the 21-day feeding bioassay in (**c**) total amounts and (**d**) relative abundances. Stress values (as a measure of "goodness-of-fit") for all NMDS were below 0.2, indicating reasonable fits (Clarke, 1993). Statistical analyses are displayed in Table 1. Orange (SAFAs), blue (MUFAs) and green (PUFAs) lines display the impact of each FA.

Diet-related diuron effects on *P. acuta*

In accordance with our hypothesis, the feeding activity and physiology of *P. acuta* was affected when fed with diuron-exposed periphyton (Fig. 5). Compared to the control, the feeding rate of snails in the diuron treatment increased over the study duration as indicated by a significant interaction of both predictor variables (i.e., *Time* × *Diuron*; Table 1 and S4). A significantly enhanced feeding rate in the diuron treatment was observed in the third week, while the feeding rate was comparable with the control during the first two weeks (Fig. 5; Table 1). This could be explained by meaningful deviations among the three independent colonization phases of periphyton resulting in variable total levels and relative abundance of FAs (Table 1), ultimately resulting in food quality variations during the course of this bioassay. Even though no significant differences were observed for phenotype-based cell groups and diatoms (Table 1), periphyton communities were probably not identical between colonization phases and therefore responded differently to diuron exposure, leading to different FA profiles of periphyton during the bioassay. An in-depth analysis of the entire microbial community structure including all important taxonomic groups (such as chlorophytes) during future studies would shed more light on periphyton responses to herbicides. Moreover, in comparison to the first two weeks, the third colonization phase showed high within-treatment variability in FAs profiles (Figure 4a, b) indicating higher differences in food quality of periphyton for *P. acuta* between control and treatment in the last week. Moreover, it is worth noting that laboratory conditions (i.e., light and nutrients) differ from periphyton colonization and the bioassay indicating that periphyton communities may have changed during the 7 days in the bioassay. Therefore, this might have an impact on the observed effects, even though the comparability is guaranteed between control and treatment due to homogeneous laboratory conditions.

Nevertheless, the higher feeding rate probably caused a significant increase in the snails' energy reserves (i.e., NLFAs) by ~65 % in the treatment (Figure 4c, Table 2) but had no significant impact on snail growth, even though a trend was observed (increased median growth rate by ~55 % compared to the control; Table 1). The increased feeding activity may be explained by pollutant-induced shifts in the community composition in favor of particular algae that ultimately increased the food quality for *P. acuta*. This assumption is supported by higher HUFA content in periphyton of the diuron treatment (by ~53 %) compared to the control, which probably resulted from higher diatom abundances. However, the total HUFA content was relatively small compared to other FA groups (contributed only ~2 % to total

FAs), pointing towards other groups of essential nutrients (e.g., amino acids; Fink et al., 2011) involved in the stimulation of feeding. An increased feeding of invertebrate grazers to compensate for nutrient deficiencies in low quality food sources as highlighted in earlier studies (Cruz-Rivera and Hay, 2000; Stelzer and Lamberti, 2002) could be also possible but seems rather unlikely in the present study. This is to be assumed, since compensatory feeding is often coupled with a lower animal growth rate due to higher energy expenditures for the increased activity (Cruz-Rivera and Hay, 2000; Plath and Boersma, 2001; Fink and Elert, 2006), which was not observed in the present study. Furthermore, the PUFA and HUFA content of periphyton exposed to diuron were higher or comparable to the control (Table S7), indicating no lower food quality for *P. acuta* in the diuron treatment. Therefore it is suggested that the increase of HUFAs (or other essential nutrients) enhanced the palatability of diuron-exposed periphyton for *P. acuta* resulting in an increased feeding rate.

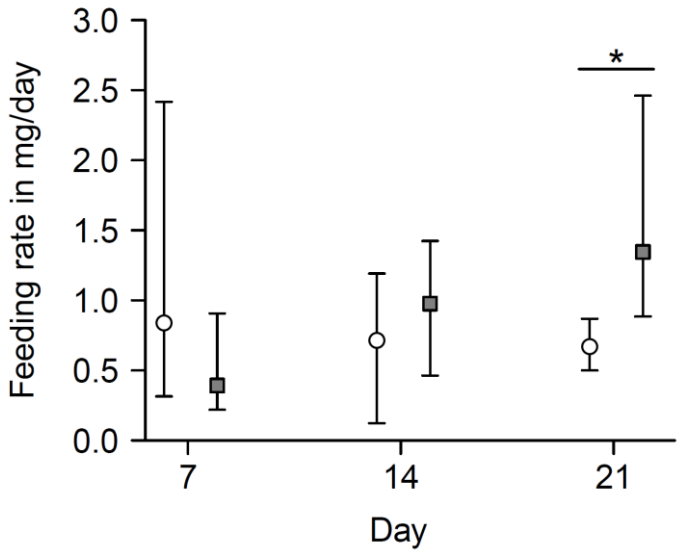

Fig. 5 Median (with 95 % CIs) feeding rate of *P. acuta* fed with periphyton colonized under control conditions (white circles; $n = 30$ at test start, $n = 28$ at day 7, $n = 27$ at day 14 and $n = 25$ at day 21) and in the presence of 8 µg diuron/L (grey squares; $n = 30$ at test start, $n = 30$ at day 7, $n = 29$ at day 14, $n = 25$ at day 21) during the 21-day feeding bioassay. The asterisk denotes a statistically significant difference to the control. Statistical analyses are displayed in Table 1.

Irrespective of the true underlying mechanism, the increased feeding rate of diuron-exposed periphyton probably provoked a faster shift in the NLFA profile reflecting the snails' aquatic diet compared to the control (Fig. 4d and Table 1). The relative abundance of most prevalent NLFA (~30 %) in the control, namely 18:1n-9 (a proxy for terrestrial plant food sources as provided prior to the start of the experiment; Gladyshev et al., 2012) was

significantly decreased in the diuron treatment (Fig. 4d; Table S8). Concurrently, relative abundances of HUFAs were significantly increased (e.g., 20:5n-3; Table S8), an indicator for aquatic food sources (Gladyshev et al., 2012). Ultimately, significantly higher PUFA reserves in snails (~125 %) fed with diuron-exposed periphyton were observed (Table S7), suggesting that these organisms had a higher physiological fitness compared to the control at the end of the bioassay (Brett et al., 2006; Menzel et al., 2018).

It cannot be excluded that diuron induced the increased feeding rate in *P. acuta* by an increasing internal exposure in snails, due to the dietary uptake of diuron-contaminated periphyton over time. In a preliminary experiment testing waterborne diuron effects on the feeding activity of *P. acuta* during 7 days, no effects were observed up to a nominal concentration of 50 mg/L (Fig. S6). Although, these results are not directly comparable, due to the different study durations, dietary diuron exposure of *P. acuta* is expected to be low during the 21-day bioassay, taking the bioaccumulation potential of diuron (European Food Safety Authority, 2005) and the concentration used in the present study into account. Based on diurons' sorption isotherms to biofilms (Chaumet et al., 2019), it was estimated that ~0.4 µg diuron accumulated per mg periphyton dry mass during each colonization. Thus, the estimated median intake of diuron by *P. acuta* during the entire study was ~12 µg (6 – 16 µg 95 % CIs) via the dietary pathway. Considering that waterborne diuron exposure of 50 mg/L over 7 days did not induce any effects on the feeding rate of the grazer, it seems rather likely that *P. acuta* were indirectly positively affected by diuron during the present study. However, the results highlight the need for further comprehensive analyses of periphyton community composition (e.g., metagenomics) and periphyton-associated macro- and micro-nutrients (e.g., nutrient stoichiometry, amino acid analysis) to gain a deeper understanding of herbicide-induced bottom-up effects on grazers.

Our study indicates that herbicides can indirectly alter the activity and physiology of grazers. Observations in the present study reflect periphyton communities likewise dominated by green algae. However, in diatom-dominated communities, negative food quality-related bottom-up effects on grazers are expected under natural conditions, since diuron contamination is known to decrease the community diversity of diatoms (Ricciardi et al., 2009). This, in turn, can have far reaching consequences for the entire food web. Herbicide-induced changes in the community composition of periphyton reduce the availability of essential nutrients (e.g., HUFAs) for invertebrate grazers, which include merolimnic insects. These alterations can be transferred to upper consumer levels that are trophically linked to

herbivorous invertebrates (Brett and Müller-Navarra, 1997). Since merolimnic aquatic organisms are generally considered a high-quality food source for terrestrial consumers in riparian ecosystems (Martin-Creuzburg et al., 2017; Moyo et al., 2017), the present study suggests implications in aquatic-terrestrial meta-ecosystems (Schulz et al., 2015). Moreover, supposed positive indirect effects in primary consumers as observed here may result in ecological surprises by unconsidered negative feedback loops: for instance, an atrazine-induced increase of periphyton and gastropod grazers enhanced parasite infections in tadpoles, as gastropods are the first intermediate host (Rohr et al., 2008). These insights highlight the need to expand the scientific understanding of direct and indirect consequences of herbicide exposures on periphyton-grazer systems to better anticipate consequences at the ecosystem level.

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Supporting information of Appendix A4

Measured temperature during the colonization by periphyton on ceramic tiles

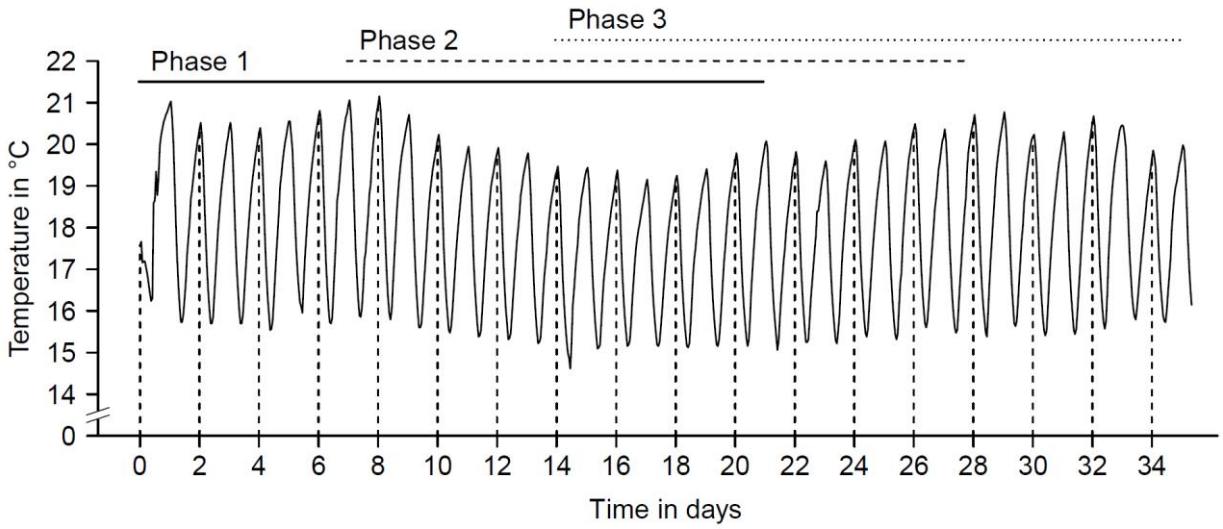

Fig. S1 Mean measured temperature during the microbial colonization phases based on three temperature loggers that continuously recorded the temperature in the water basin.

Results of stain-free flow cytometry and visual stochastic network embedding (viSNE) analysis

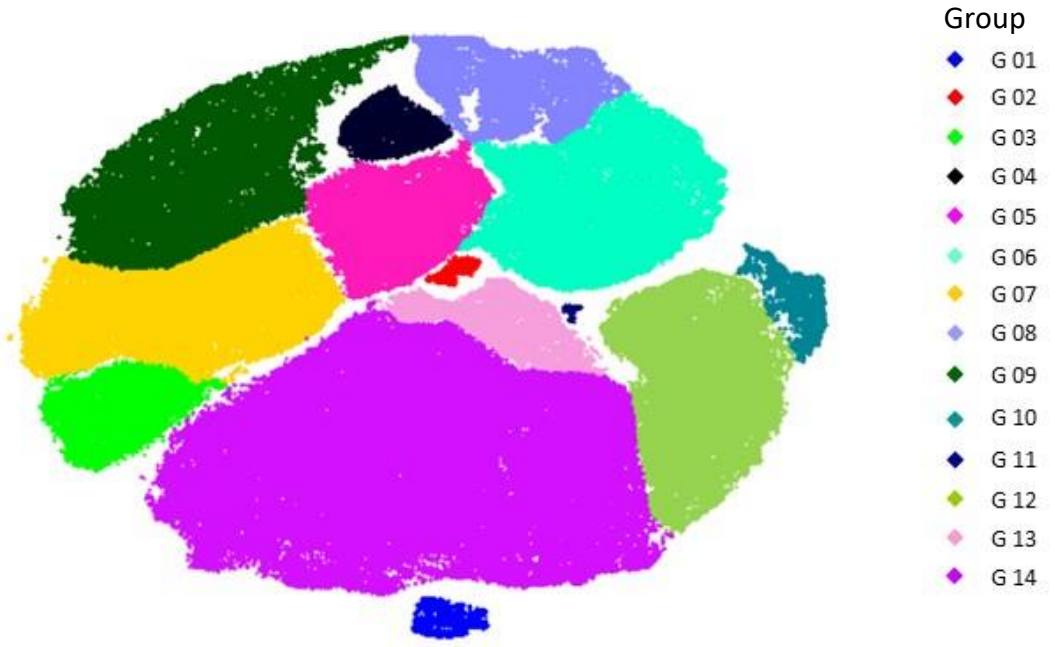

Fig. S2 Phenotype-based cell groups (G 01 – G 14) created via viSNE analysis that is based on optical scatter and fluorescence intensities (for more details, see 2.5.2 Microbial community structure and cell viability in main text).

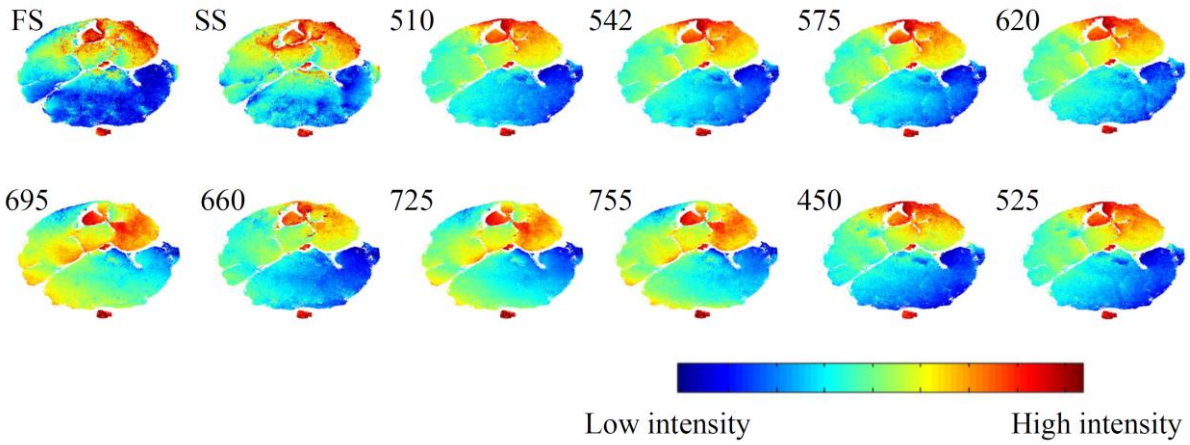

Fig. S3 Colored viSNE map of the forward (FS) and side scatter (SS) and the 10 fluorescence parameters (fluorescence emission from 450 – 755 nm) from **Fig. S2**.

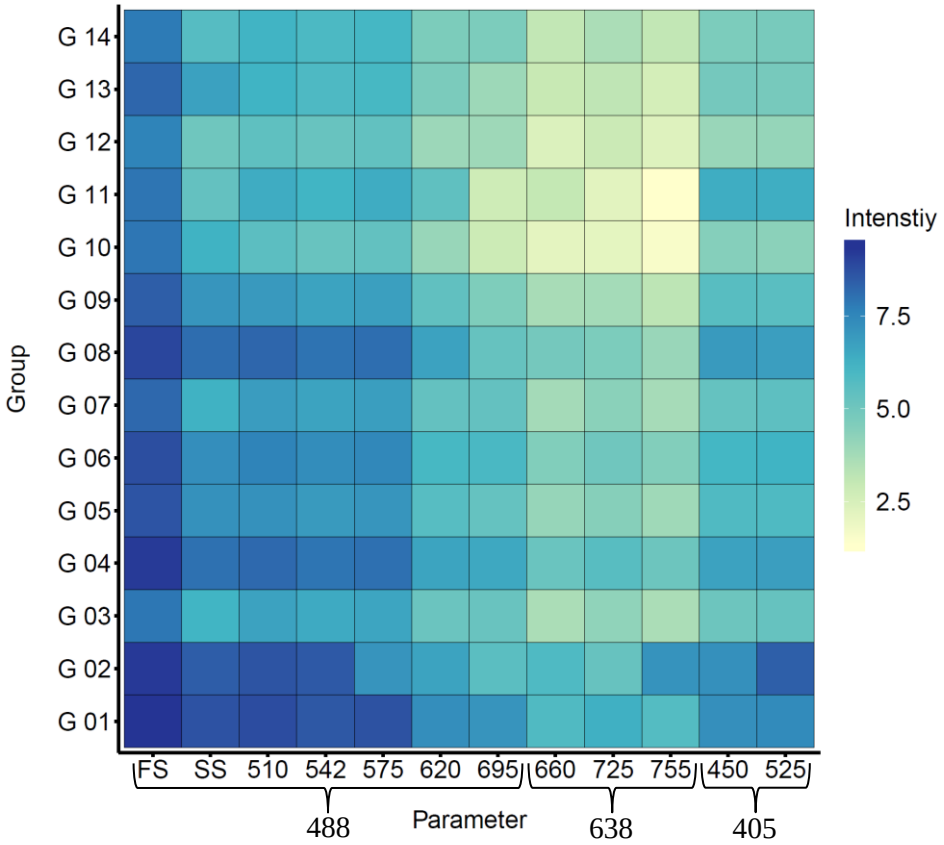

Fig. S4 Heatmap of the 12 parameters (see **Figure S3** for more details) of each phenotype-based group (cf. **Figure S2)** measured via the blue (488 nm), red (638 nm) and violet laser (405 nm). The blue laser serves for identification of diatom/green algae groups as well as decaying cells, the red laser for the identification of cyanobacteria groups and the violet laser for the identification of decaying cell groups. None of the clusters shows a fluorescence pattern previously found in cyanobacteria (Sgier et al., 2016). Groups 01 – 09 show properties of diatom/green algae-like cells, while groups 10 – 14 show properties of decaying cells.Sgier et al., 2016 In comparison to other data sets, cells with strong forward scatter have been detected (FS), a proxy for cell size.

Grazed periphyton area by *P. acuta* after 7 days

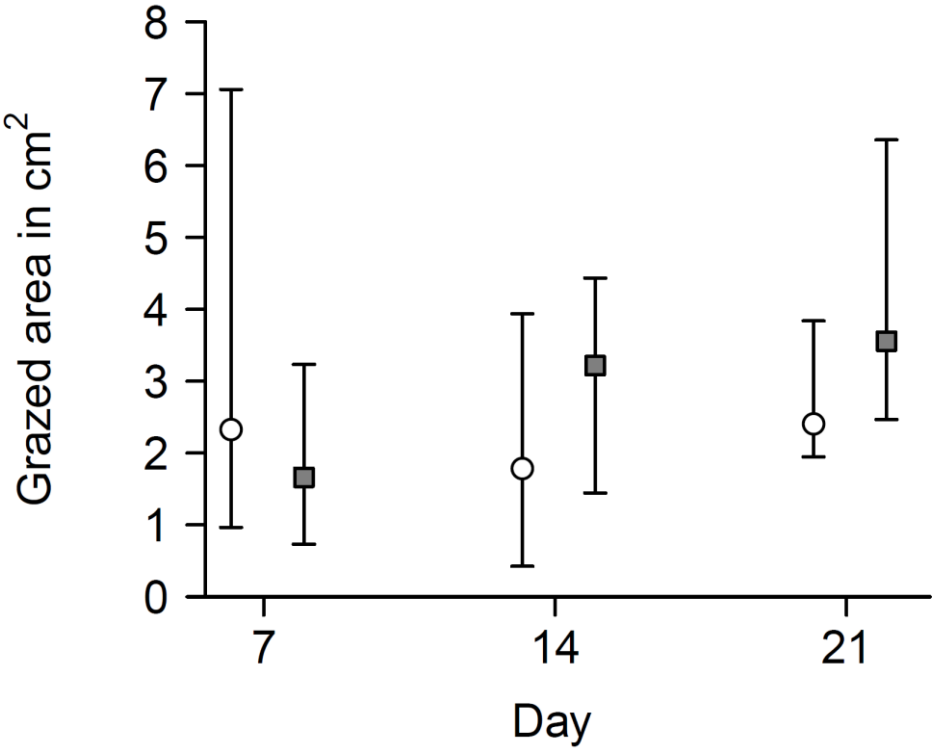

Fig. S5 Median (with 95 % CIs) grazed area by *P. acuta* after 7 days fed with periphyton colonized under control conditions (white circles; n = 30 at test start, n = 28 at day 7, n = 27 at day 14 and n = 25 at day 21) and in the presence of 8 µg diuron/L (grey squares; n = 30 at test start, n = 30 at day 7, n = 29 at day 14, n = 25 at day 21) during the 21-day feeding bioassay. Statistical analyses are displayed in Table S3.

Table S1 Algae medium according to Kuhl and Lorenzen (1964).

Compound	Concentration (mg/L)
KNO_3	1011.1
$NaHPO_4 \times H_2O$	621.0
Na_2HPO_4	71.0
$MgSO_4 \times 7H_2O$	246.5
$CaCl_2 \times 2H_2O$	14.7
$FeSO_4 \times 2H_2O$	7.0
$Na_2EDTA \times H_2O$	9.3
H_3BO_3	0.061
$MnSO_4 \times H_2O$	0.169
$ZnSO_4 \times 7H_2O$	0.287
$CuSO_4 \times 5H_2O$	0.0025
$Na_2MoO_4 \times 2H_2O$	0.017

Table S2 Mean abiotic parameters (± SE) measured in the stream channels during the microbial colonization.

Phase	Treatment	pH	O_2 (mg/L)	EC (mS/cm)	*v* (m/s)	PPFD (µmol/m²s)
P1	Control	8.12 (±0.12)	9.96 (±0.59)	2.52 (±0.06)	0.13 (±0.01)	91.00 (±6.08)
		8.84 (±0.34)	10.38 (±0.63)	2.56 (±0.09)	0.14 (±0.01)	204.00 (±13.58)
		8.72 (±0.27)	10.92 (±1.06)	2.56 (±0.10)	0.10 (±0.01)	161.33 (±9.61)
	Diuron	8.14 (±0.11)	10.13 (±0.63)	2.55 (±0.07)	0.12 (±0.01)	112.67 (±7.97)
		8.32 (±0.16)	10.15 (±0.63)	2.61 (±0.09)	0.10 (±0.00)	158.67 (±9.68)
		8.51 (±0.23)	10.42 (±0.71)	2.60 (±0.08)	0.12 (±0.01)	163.33 (±8.19)
P2	Control	8.83 (±0.35)	11.29 (±1.03)	2.37 (±0.11)	0.12 (±0.01)	175.67 (±6.36)
		9.36 (±0.52)	10.79 (±1.34)	2.38 (±0.12)	0.13 (±0.01)	226.00 (±11.68)
		8.87 (±0.34)	10.67 (±1.03)	2.46 (±0.13)	0.14 (±0.02)	193.33 (±7.13)
	Diuron	8.38 (±0.19)	10.66 (±0.80)	2.38 (±0.11)	0.13 (±0.01)	181.33 (±32.10)
		8.86 (±0.34)	10.73 (±1.09)	2.41 (±0.11)	0.09 (±0.01)	221.00 (±5.51)
		8.51 (±0.24)	10.66 (±0.98)	2.44 (±0.11)	0.14 (±0.01)	188.67 (±37.02)
P3	Control	8.73 (±0.25)	12.63 (±0.44)	2.36 (±0.09)	0.11 (±0.02)	197.00 (±7.55)
		9.29 (±0.42)	13.07 (±0.75)	2.34 (±0.10)	0.10 (±0.01)	200.00 (±13.53)
		8.60 (±0.18)	12.02 (±0.36)	2.36 (±0.11)	0.14 (±0.01)	193.00 (±16.04)
	Diuron	8.91 (±0.30)	12.21 (±0.37)	2.35 (±0.11)	0.14 (±0.01)	225.67 (±11.68)
		8.92 (±0.27)	12.66 (±0.53)	2.34 (±0.12)	0.14 (±0.01)	214.67 (±28.10)
		8.25 (±0.07)	11.53 (±0.25)	2.28 (±0.08)	0.15 (±0.02)	147.67 (±6.36)

Phase (microbial conditioning phase); O_2 (oxygen); EC (electrical conductivity); *v* (velocity); PPFD (photon flux density)

Table S3 Mean measured diuron concentrations (with 95 % CIs) for the stream channels during the microbial colonization.

Treatment	LCL (µg/L)	*n*	Fresh medium (µg/L)	3-day-old medium (µg/L)
Control	1.00	3	<LCL	-
Diuron		8	9.26 (8.55 to 9.97)	7.32 (6.47 to 8.17)

LCL (lowest calibration level); *n* (number of replicates)

Table S4 Results of multilevel model analysis for the feeding activity of *P. acuta* (in form of the proxy the snais' feeding rate and grazed area by *P. acuta*) during the 21-day feeding bioassay.

Endpoint	Model	AIC	*Predictor*	Parameter estimate (±95 % CI)	df	*t*-value	*p*-value	Compared to model	Likelihood ratio test
Feeding rate	1	165	*(Intercept)*	-0.194 (-0.290 to -0.098)	106	-3.986	<0.001		
in mg/d	2	165	*(Intercept)*	-0.275 (-0.424 to -0.126)	105	-3.642	<0.001		
(log-transformed)			*Time*	0.006 (-0.002 to 0.014)	105	1.399	0.165	1	0.161
	3	167	*(Intercept)*	-0.307 (-0.485 to -0.128)	105	-3.376	0.001		
			Time	0.006 (-0.002 to 0.015)	105	1.403	0.164		
			Diuron	0.061 (-0.131 to 0.252)	56	0.627	0.533	2	0.528
	4	147	*(Intercept)*	-0.045 (-0.247 to 0.156)	104	-0.440	0.661		
			Time	-0.013 (-0.024 to -0.002)	104	-2.392	0.019		
			Diuron	-0.447 (-0.731 to -0.164)	56	-3.120	0.003		
			Time× Diuron	0.038 (0.022 to 0.053)	104	4.806	<0.001	3	<0.001
Grazed area	1	149	*(Intercept)*	-0.178 (-0.275 to -0.080)	106	-3.592	<0.001		
after 7 d in cm^2	2	149	*(Intercept)*	-0.256 (-0.398 to -0.113)	105	-3.524	0.001		
(log-transformed)			*Time*	0.006 (-0.002 to 0.014)	105	1.462	0.147	1	0.143
	3	151	*(Intercept)*	-0.283 (-0.458 to -0.109)	105	-3.188	0.002		
			Time	0.006 (-0.002 to 0.014)	105	1.464	0.146		
			Diuron	0.053 (-0.142 to 0.248)	56	0.540	0.592	2	0.587
	4	144	*(Intercept)*	-0.124 (-0.325 to 0.076)	104	-1.215	0.227		
			Time	-0.006 (-0.017 to 0.005)	104	-1.094	0.276		
			Diuron	-0.257 (-0.539 to 0.025)	56	-1.804	0.077		
			Time× Diuron	0.023 (0.008 to 0.038)	104	3.010	0.003	3	0.003

AIC (Akaike's information criterion); Predictor (predictor variables; see text for more details) Parameter estimate with ±95 % CI (represents the effect direction of each predictor variable); df (degrees of freedom); *t*-value (value of *t*-statistic); *p*-value (< 0.05 indicate a relationship between predictor and response variable in the respective model); Likelihood ratio test (compares multilevel models by stepwise adding predictor variables and thus statistically evaluates the impact of each predictor on the response variable; $p < 0.05$ indicates a statistically significant impact of the predictor variable)

Table S5 Medians (with 95 % CIs) of the relative abundance in % of phenotype-based cell groups analyzed during the study. Moreover, statistical tests used as well as *p*-values from statistical comparisons of the diuron treatment with the control are displayed. All *p*-values below 0.05 are printed in bold.

Group	Treatment	Median	±95 % CI	Statistical test	*p*-value
G 01	Control	0.37	0.27 to 0.67	Wilcoxon rank-sum	
	Diuron	0.78	0.44 to 1.33		0.094
G 02	Control	0.22	0.15 to 0.48	Wilcoxon rank-sum	
	Diuron	0.51	0.40 to 0.87		**0.019**
G 03	Control	4.99	3.50 to 6.32	Wilcoxon rank-sum	
	Diuron	1.47	0.90 to 3.55		**0.006**
G 04	Control	1.93	1.75 to 2.77	Student's *t*	
	Diuron	2.33	1.79 to 3.60		0.464
G 05	Control	4.92	4.16 to 5.83	Student's *t*	
	Diuron	8.32	5.17 to 10.26		**0.014**
G 06	Control	11.57	8.48 to 13.31	Student's *t*	
	Diuron	10.28	7.68 to 13.05		0.502
G 07	Control	18.05	13.97 to 20.62	Wilcoxon rank-sum	
	Diuron	4.14	3.36 to 12.51		**<0.001**
G 08	Control	0.26	0.13 to 0.41	Wilcoxon rank-sum	
	Diuron	5.28	1.61 to 19.42		**0.001**
G 09	Control	3.07	2.25 to 4.84	Wilcoxon rank-sum	
	Diuron	13.28	6.59 to 23.00		**0.004**
G 10	Control	0.37	0.29 to 0.62	Wilcoxon rank-sum	
	Diuron	1.33	0.30 to 3.95		0.077
G 11	Control	0.1	0.01 to 0.19	Wilcoxon rank-sum	
	Diuron	0.05	0.01 to 0.12		0.387
G 12	Control	11.68	7.99 to 13.85	Wilcoxon rank-sum	
	Diuron	9.69	4.33 to 13.88		0.436
G 13	Control	0.81	0.57 to 1.32	Wilcoxon rank-sum	
	Diuron	4.73	1.30 to 9.07		**0.008**
G 14	Control	40.17	38.36 to 44.04	Wilcoxon rank-sum	
	Diuron	33.62	12.97 to 45.13		0.113

Table S6 Medians (with 95 % CIs) of the relative sequence abundance in % of diatom species analyzed during the study. Medians and most of the respective confidence intervals of the majority of diatom species are zero indicating that most species were relatively rare in the periphyton communities of the present study. Moreover, since the dataset contains too many zeros (i.e., identical values), statistical comparisons with the respective control (via Wilcoxon rank-sum test) were not performed as *p*-values would not be reliable.

Diatom species	Treatment	Median	±95 % CI
Achnanthidium minutissimum	Control	0.00	0.00 to 0.04
	Diuron	0.00	0.00 to 0.05
Achnanthidium rivulare	Control	0.00	0.00 to 0.00
	Diuron	0.00	0.00 to 0.00
Cocconeis sp.	Control	0.00	0.00 to 0.00
	Diuron	0.00	0.00 to 0.00
Cyclotella meneghiniana	Control	0.00	0.00 to 0.00
	Diuron	0.00	0.00 to 0.02
Denticula tenuis	Control	0.00	0.00 to 0.00
	Diuron	0.00	0.00 to 0.00
Epithemia parallela	Control	0.00	0.00 to 0.00
	Diuron	0.00	0.00 to 0.00
Fistulifera saprophila	Control	0.00	0.00 to 0.02
	Diuron	0.00	0.00 to 0.00
Gomphonema acuminatum	Control	0.00	0.00 to 0.00
	Diuron	0.00	0.00 to 0.02
Gomphonema affine	Control	0.04	0.02 to 0.09
	Diuron	0.20	0.13 to 0.49
Gomphonema carolinense	Control	0.00	0.00 to 0.01
	Diuron	0.00	0.00 to 0.00
Gomphonema hebridense	Control	0.20	0.03 to 0.48
	Diuron	0.22	0.09 to 0.73
Gomphonema parvulum	Control	5.20	3.70 to 9.51
	Diuron	12.11	3.49 to 29.47
Gomphonema productum	Control	0.08	0.02 to 0.17
	Diuron	0.31	0.04 to 2.25
Gomphonema rhombicum	Control	0.00	0.00 to 0.01
	Diuron	0.00	0.00 to 0.00
Gomphonema rosenstockianum	Control	0.00	0.00 to 0.03
	Diuron	0.00	0.00 to 0.00
Gomphonema saprophilum	Control	0.17	0.07 to 0.26
	Diuron	0.48	0.04 to 0.92
Lemnicola hungarica	Control	0.00	0.00 to 0.00
	Diuron	0.00	0.00 to 0.00
Mayamaea permitis	Control	1.55	0.29 to 4.25
	Diuron	3.21	0.18 to 5.63
Nitzschia acidoclinata	Control	0.28	0.08 to 0.33
	Diuron	1.07	0.16 to 2.38

Table S6 continued.

Diatom species	Treatment	Median	±95 % CI
Nitzschia communis	Control	0.61	0.24 to 1.27
	Diuron	0.04	0.00 to 0.16
Nitzschia fonticola	Control	0.00	0.00 to 0.02
	Diuron	0.05	0.00 to 0.16
Nitzschia palea	Control	90.67	81.73 to 93.26
	Diuron	70.32	43.96 to 88.98
Nitzschia pusilla	Control	0.00	0.00 to 0.02
	Diuron	0.00	0.00 to 0.37
Pinnularia sinistra	Control	0.00	0.00 to 0.00
	Diuron	0.00	0.00 to 0.00
Planothidium frequentissimum	Control	0.00	0.00 to 0.00
	Diuron	0.00	0.00 to 0.02
Planothidium lanceolatum	Control	0.00	0.00 to 0.00
	Diuron	0.00	0.00 to 0.00
Sellaphora minima	Control	0.04	0.00 to 0.16
	Diuron	0.27	0.00 to 0.87
Sellaphora seminulum	Control	1.24	0.79 to 2.30
	Diuron	4.03	2.54 to 13.15
Stauroneis kriegeri	Control	0.00	0.00 to 0.00
	Diuron	0.00	0.00 to 0.07
Staurosira shiloi	Control	0.03	0.00 to 0.06
	Diuron	0.13	0.00 to 0.25
Surirella brebissonii	Control	0.00	0.00 to 0.00
	Diuron	0.00	0.00 to 0.00
Ulnaria ulna	Control	0.00	0.00 to 0.00
	Diuron	0.00	0.00 to 0.00

Table S7 Median (with 95 % CIs) FAs and NLFAs in µg/mg of periphyton and snails, respectively, analyzed during the study. Endpoints were statistically evaluated using pairwise comparison (Students'*t* and Wilcoxon rank-sum test). Values printed in bold indicate a statistically significant difference relative to the control and asterisks denote *p*-values below 0.05 (*), 0.01 (**) and 0.001 (***).

Fatty acid(s)	FAs of Periphyton		NLFAs of Snails	
	Control	Diuron	Control	Diuron
12:0	-	-	0.01 (0.01 to 0.02)	**0.02 (0.01 to 0.03)****
14:0	-	-	0.03 (0.03 to 0.05)	**0.26 (0.24 to 0.31)*****
15:0	0.04 (0.02 to 0.05)	0.05 (0.04 to 0.07)	0.01 (0.01 to 0.02)	**0.05 (0.03 to 0.12)****
16:0	3.95 (2.26 to 4.77)	**5.00 (4.42 to 6.78)***	1.41 (0.85 to 2.35)	**2.34 (2.13 to 3.59)****
17:0	0.80 (0.40 to 0.93)	0.87 (0.59 to 1.11)	0.12 (0.08 to 0.17)	**0.21 (0.19 to 0.30)*****
18:0	0.11 (0.09 to 0.15)	**0.17 (0.15 to 0.40)****	0.59 (0.55 to 0.66)	**0.99 (0.79 to 1.45)****
20:0	0.00 (0.00 to 0.01)	0.00 (0.00 to 0.01)	0.01 (0.01 to 0.02)	**0.02 (0.01 to 0.03)****
22:0	0.02 (0.01 to 0.03)	**0.01 (0.00 to 0.02)***	0.01 (0.01 to 0.02)	**0.03 (0.02 to 0.03)****
23:0	0.02 (0.01 to 0.03)	0.02 (0.02 to 0.03)	0.02 (0.02 to 0.03)	**0.05 (0.04 to 0.09)*****
24:0	0.04 (0.02 to 0.05)	0.03 (0.02 to 0.05)	0.02 (0.01 to 0.03)	0.02 (0.01 to 0.04)
SAFAs	4.97 (1.46 to 6.14)	**6.12 (5.28 to 7.60)***	2.26 (1.58 to 3.36)	**4.05 (3.70 to 5.93)****
16:1n-7	0.53 (0.26 to 0.91)	**1.24 (0.97 to 1.59)****	0.10 (0.09 to 0.11)	**0.46 (0.35 to 0.68)****
18:1n-7	1.71 (1.01 to 2.25)	**2.72 (2.21 to 3.21)****	0.32 (0.26 to 0.33)	**1.00 (0.78 to 1.86)****
18:1n-9	0.88 (0.64 to 0.97)	**1.13 (1.02 to 1.28)****	2.08 (0.99 to 4.21)	1.06 (0.94 to 1.58)
20:1n-9	0.01 (0.01 to 0.01)	0.01 (0.00 to 0.01)	0.05 (0.05 to 0.06)	**0.11 (0.09 to 0.17)****
22:1n-9	-	-	0.01 (0.00 to 0.03)	0.02 (0.00 to 0.02)
MUFAs	3.19 (1.02 to 4.57)	**5.27 (4.50 to 5.72)*****	2.54 (1.47 to 4.66)	2.62 (2.18 to 4.27)
18:2n-6	1.88 (1.22 to 2.21)	2.11 (1.67 to 3.12)	0.49 (0.43 to 0.66)	**1.24 (1.09 to 1.86)****
18:3n-3	7.61 (4.56 to 8.72)	8.30 (6.59 to 9.92)	0.71 (0.40 to 1.01)	**1.43 (1.32 to 2.25)****
18:3n-6	0.47 (0.22 to 0.88)	0.66 (0.34 to 0.87)	0.01 (0.01 to 0.01)	**0.02 (0.02 to 0.04)*****
20:2n-6	0.01 (0.01 to 0.01)	0.01 (0.01 to 0.02)	0.10 (0.08 to 0.14)	**0.25 (0.16 to 0.52)****
20:3n-3	0.01 (0.00 to 0.01)	**0.02 (0.01 to 0.05)****	0.03 (0.02 to 0.05)	**0.07 (0.04 to 0.14)****
20:3n-6	-	-	0.02 (0.01 to 0.06)	**0.12 (0.07 to 0.25)****
20:4n-6	0.03 (0.02 to 0.05)	**0.10 (0.06 to 0.19)****	0.43 (0.28 to 0.59)	**0.78 (0.53 to 2.01)***
20:5n-3	0.22 (0.13 to 0.37)	0.31 (0.21 to 0.63)	0.10 (0.07 to 0.12)	**0.30 (0.21 to 0.54)****
22:6n-3	0.05 (0.03 to 0.07)	0.05 (0.03 to 0.09)	0.02 (0.01 to 0.02)	**0.04 (0.03 to 0.10)***
PUFAs	10.27 (2.64 to 13.19)	11.79 (9.24 to 15.31)	1.90 (1.80 to 2.20)	**4.31 (3.71 to 7.70)****

Table S8 Median (with 95 % CIs) FA and NLFA composition in % of periphyton and snails, respectively, analyzed during the study. Endpoints were statistically evaluated using pairwise comparison (Students'*t* and Wilcoxon rank-sum test). Values printed in bold indicate a statistically significant difference relative to the control and asterisks denote *p*-values below 0.05 (*), 0.01 (**) and 0.001 (***).

Fatty acid(s)	FAs of Periphyton		NLFAs of Snails	
	Control	Diuron	Control	Diuron
12:0	-	-	0.17 (0.09 to 0.20)	0.20 (0.07 to 0.25)
14:0	-	-	0.49 (0.36 to 0.72)	**2.36 (1.71 to 2.44)****
15:0	0.22 (0.16 to 0.27)	0.19 (0.18 to 0.27)	0.20 (0.11 to 0.24)	**0.39 (0.32 to 1.24)****
16:0	21.69 (19.34 to 23.55)	22.16 (20.9 to 25.18)	21.01 (17.40 to 22.96)	21.03 (20.05 to 23.66)
17:0	3.79 (3.16 to 4.87)	3.68 (2.66 to 4.65)	1.71 (1.59 to 1.77)	1.96 (1.65 to 2.12)
18:0	0.66 (0.56 to 1.12)	0.82 (0.63 to 1.43)	8.87 (6.48 to 11.34)	8.38 (7.61 to 10.62)
20:0	0.01 (0.01 to 0.04)	0.02 (0.01 to 0.03)	0.17 (0.13 to 0.22)	0.17 (0.14 to 0.31)
22:0	0.18 (0.05 to 0.23)	**0.04 (0.02 to 0.08)***	0.21 (0.16 to 0.22)	0.23 (0.14 to 0.30)
23:0	0.10 (0.06 to 0.18)	0.08 (0.07 to 0.12)	0.33 (0.18 to 0.45)	**0.49 (0.41 to 0.61)****
24:0	0.25 (0.14 to 0.36)	0.14 (0.10 to 0.25)	0.25 (0.20 to 0.32)	0.21 (0.12 to 0.43)
SAFAs	27.18 (23.47 to 31.60)	27.16 (25.71 to 31.59)	33.03 (32.46 to 34.20)	**36.53 (33.12 to 39.33)****
16:1n-7	3.33 (1.97 to 4.35)	**5.21 (3.75 to 7.53)***	1.44 (1.10 to 1.85)	**3.94 (3.48 to 5.30)*****
18:1n-7	9.40 (7.71 to 11.27)	**11.78 (10.57 to 12.42)***	4.52 (2.59 to 6.86)	**8.73 (7.84 to 10.40)*****
18:1n-9	4.73 (4.34 to 6.77)	4.95 (4.09 to 5.99)	31.06 (20.27 to 41.12)	**9.51 (8.57 to 12.97)****
20:1n-9	0.05 (0.04 to 0.07)	**0.04 (0.02 to 0.05)***	0.72 (0.60 to 1.05)	**0.93 (0.91 to 1.17)***
22:1n-9	-	-	0.12 (0.06 to 0.36)	0.12 (0.04 to 0.16)
MUFAs	17.57 (16.50 to 21.01)	**22.81 (18.45 to 24.89)****	37.91 (30.10 to 45.57)	**23.72 (21.75 to 28.06)*****
18:2n-6	10.55 (9.46 to 11.58)	10.28 (7.86 to 12.13)	7.34 (6.43 to 9.13)	**10.69 (9.77 to 12.55)*****
18:3n-3	39.51 (35.85 to 42.61)	**34.64 (31.73 to 37.2)***	9.85 (8.24 to 10.82)	**12.89 (11.63 to 14.59)*****
18:3n-6	2.64 (1.75 to 4.11)	2.85 (1.57 to 3.50)	0.11 (0.08 to 0.14)	**0.21 (0.16 to 0.23)*****
20:2n-6	0.05 (0.04 to 0.07)	0.05 (0.03 to 0.07)	1.52 (0.89 to 2.80)	2.36 (1.67 to 2.89)
20:3n-3	0.04 (0.03 to 0.06)	**0.11 (0.06 to 0.22)***	0.39 (0.17 to 1.00)	0.59 (0.46 to 0.76)
20:3n-6	-	-	0.37 (0.13 to 1.13)	**1.11 (0.69 to 1.37)***
20:4n-6	0.22 (0.13 to 0.26)	**0.37 (0.24 to 0.98)***	6.42 (3.13 to 12.03)	7.51 (5.37 to 11.21)
20:5n-3	1.35 (0.88 to 2.00)	1.38 (0.85 to 3.34)	1.50 (0.70 to 2.53)	**2.62 (2.13 to 3.15)****
22:6n-3	0.27 (0.21 to 0.35)	0.18 (0.12 to 0.38)	0.25 (0.15 to 0.46)	0.36 (0.27 to 0.53)
PUFAs	54.89 (51.14 to 59.88)	**50.74 (47.81 to 52.32)****	28.50 (21.55 to 37.44)	**39.71 (34.91 to 43.01)****

Preliminary experiment: 7-day feeding activity bioassay with *Physella acuta*

Before the start of the 21-day feeding bioassay, a preliminary test was performed assessing waterborne diuron effects on the survival and feeding rate (in mg/mg snail dry mass/d) of *P. acuta* using a 7-day feeding activity bioassay. This bioassay was performed in August 2018, and followed the well-established protocol by Zubrod et al. (2014).

Briefly, dried leaf discs from *Alnus glutinosa* (Ø = 20 mm), that were microbially conditioned and autoclaved before, were weighed to the nearest 0.01 mg and re-soaked for 48 hours in snail culture medium SAM-5S by Borgmann (1996). Subsequently, 10 snails (shell length of 6 – 8 mm) per nominal test concentration (i.e., 0, 12.5, 25, and 50 mg diuron/L) were fed with two pre-weighed leaf discs for 7 days under waterborne diuron exposure and laboratory conditions as described for the 21-day feeding bioassay (see main text). At the end of the bioassay, leaf disc remains were dried and weighed to the nearest 0.01 mg. No snail died during the study. Feeding rate was calculated as per Zubrod et al. (2014) and statistically analyzed via Wilcoxon rank-sum test. No statistically significant effects (p value < 0.05) were observed for the response variable feeding rate even at the highest test concentration suggesting that *P. acuta* is less sensitive to waterborne diuron exposure.

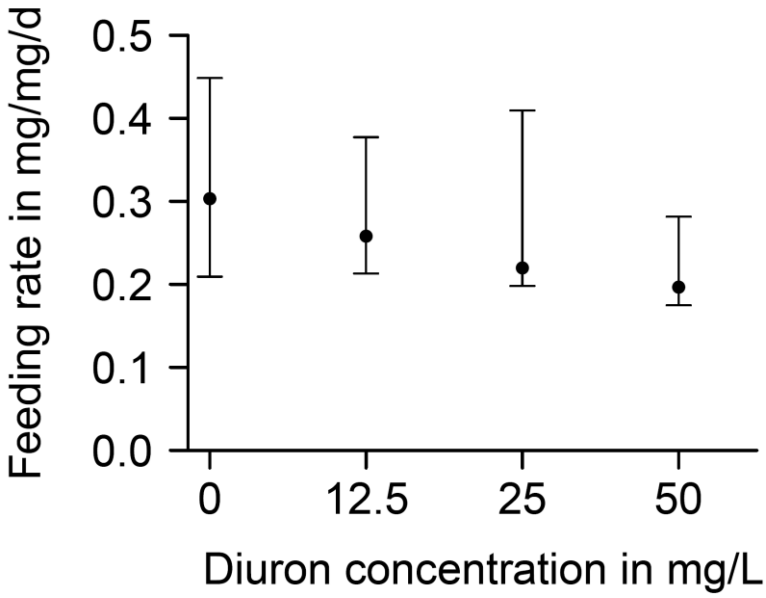

Fig. S6 Median feeding rate (black circles with 95 % CIs) of *P. acuta* when exposed to increasing nominal diuron concentrations.

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Appendix A5

Curriculum Vitae

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Education & Career

Since 06/2020	**Research Associate** Department of Plant Protection Umweltbundesamt (UBA), Dessau-Roßlau
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10/2008 – 12/2015	**University Student** Studies in Environmental Sciences Diploma Thesis: Einfluss organischer und anorganischer Fungizide auf die Fraßaktivität und das Fraßwahlverhalten von *Chaetopteryx villosa* (Trichoptera) University of Koblenz-Landau, Campus Landau

08/2007 – 05/2008 **Community Service**

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06/2004 – 06/2007 **Secondary School Student**

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Publication List (Peer-Reviewed Articles)

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