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Research paper

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.

1. 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

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.

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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 and 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.

2. Material and methods

2.1. 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, 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.

2.2. 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.

2.3. 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, 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.

2.4. 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.

2.5. 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 3 N 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.

2.6. 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 h 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.

2.7. Fungicide analyses

Nominal AZO concentrations were verified by taking samples from each 15-L aquarium at the start of the conditioning and after 3 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

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.

Concentration (μg/L)	Endpoint	Added factorial predictor	Model	ΔAIC	Com-pared 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 (rank transformed)	–	1						
		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 (log-transformed)	–	1						
		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		

concentrations (Table S1), nominal concentrations are reported throughout this manuscript.

2.8. 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

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	45,016	–	37.765	**< 0.001**	[a]Two-way
	Diet	1	1702	–	1.428	0.234	ANOVA
	Water × *Diet*	1	6192	–	5.195	**0.024**	(rank trans-
	Residuals	132	157,344	–			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	12,775	12,775	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	28,652	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			

[a] Due to unbalanced data, Type II instead of Type I sums of squares were used (Langsrud, 2003).

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	1,160,942	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 composition of gammarids	*Water*	1	0.003972	0.01335	0.491	0.736	PERMANOVA
	Diet	1	0.005252	0.01766	0.649	0.604	(square root
	Water × *Diet*	1	0.004957	0.01667	0.613	0.615	transformed)
	Residuals	35	0.283244	0.95232			

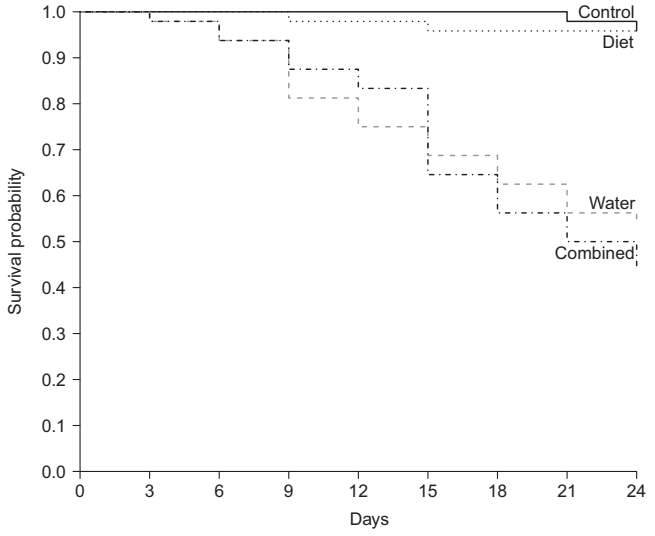

Fig. 1. Kaplan-Meier survival plot for *G. fossarum* subjected to the Control (black solid line), Water (light gray dashed line), Diet (dark gray dotted line) and Combined (black dot-dashed line) treatment during the 24-day bioassay with 30 µg AZO/L (see for Kaplan-Meier estimates Table S3). Statistical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

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.

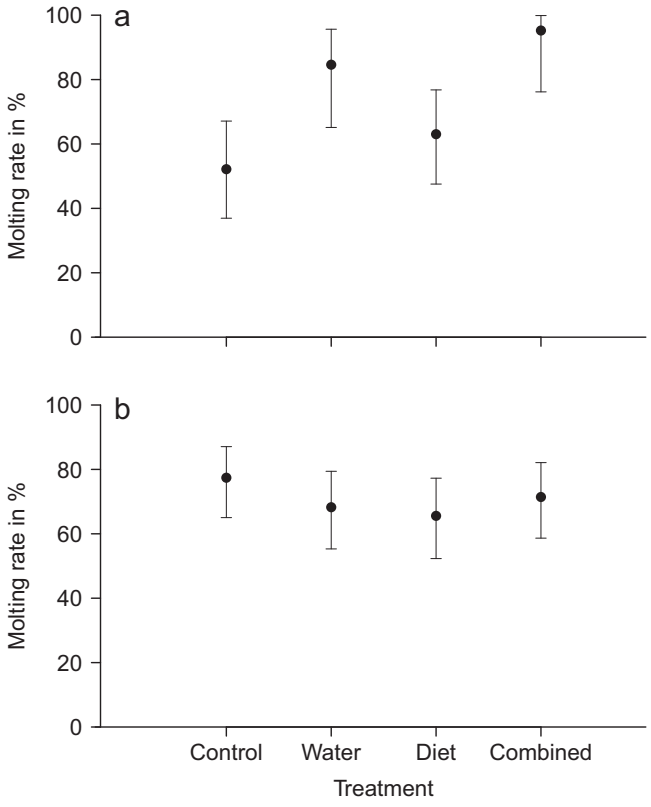

Fig. 2. Rate of molted individuals of surviving gammarids (with 95% CIs) subjected to different effect pathways during the 24-day bioassays with 30 (a) and 15 (b) µg AZO/L. Statistical analyses are displayed in Table S2.

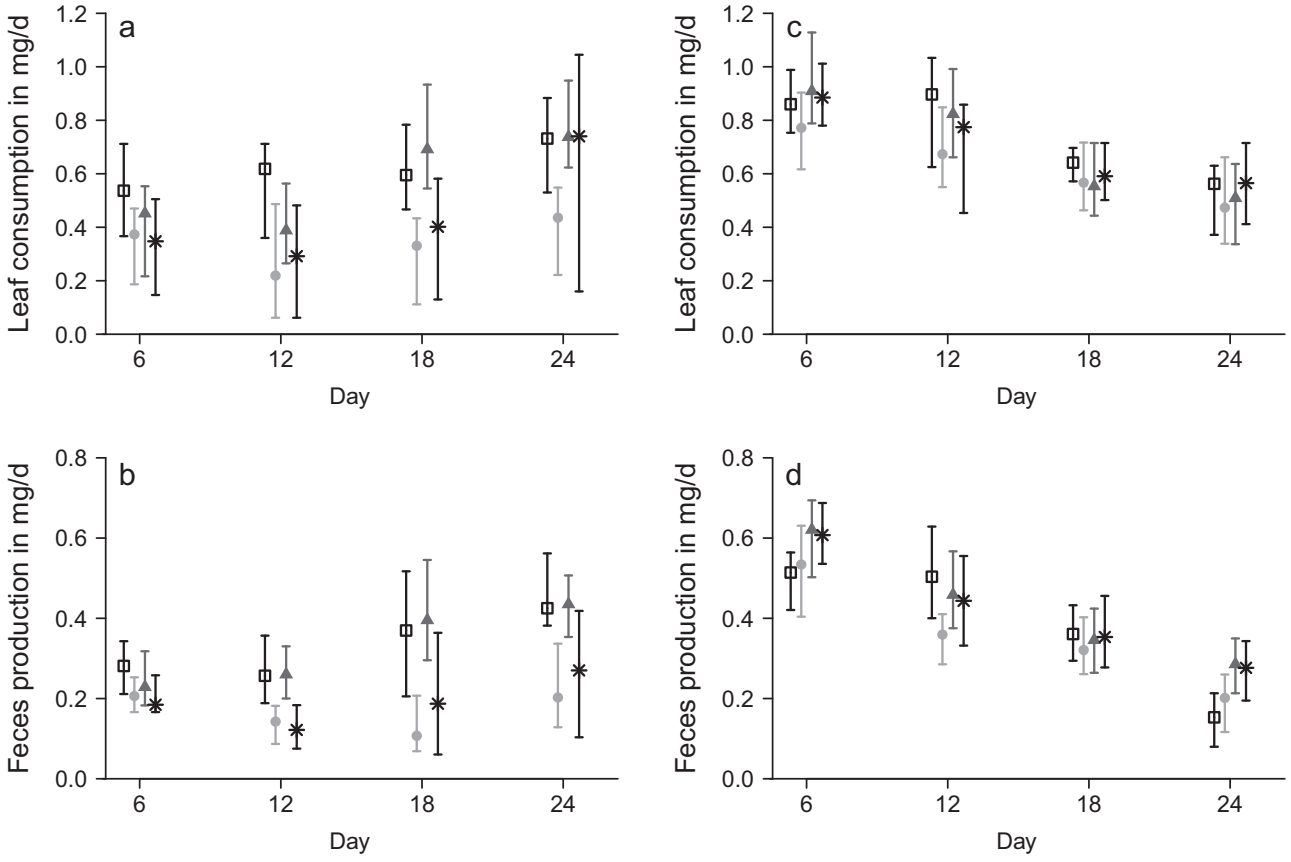

Fig. 3. Median (with 95% CIs) leaf consumption and feces production of *G. fossarum* subjected to the Control (black square), Water (light gray dot), Diet (dark gray triangle) and Combined (black asterisk) treatments during the 24-day bioassays with 30 (a and b) and 15 (c and d) μg AZO/L. Statistical analyses are displayed in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

3.1. 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; Tables S2 and 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.

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). 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, 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

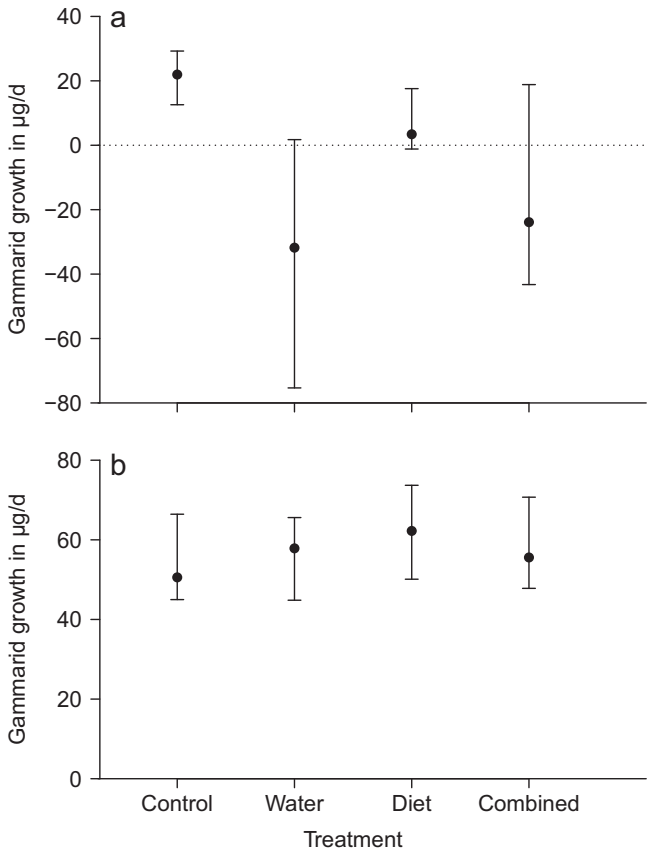

Fig. 4. Median (with 95% CIs) growth rate of *G. fossarum* subjected to different effect pathways during the 24-day bioassays with 30 (a) and 15 (b) µg AZO/L. Statistical analyses are displayed in Table 2.

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).

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.

3.2. 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 (Figs. 2b, 3c, 3d and 4b; Tables 1 and 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).

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 (Tables 1, 3 and 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 and 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.

4. 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.

CRediT authorship contribution statement

M. Konschak: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization, Project administration **J.P. Zubrod:** Conceptualization, Writing - review & editing, Supervision **P. Baudy:** Conceptualization, Investigation **P. Fink:** Methodology, Writing - review & editing, Resources **K.G.J. Kenngott:** Conceptualization, Methodology, Resources **D. Englert:** Investigation **N. Röder:** Investigation **C. Ogbeide:** Investigation **R. Schulz:** Conceptualization, Resources, Writing - review & editing, Funding acquisition **M. Bundschuh:** Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111848.

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