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*, gammarids' leaf consumption 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 gammarids' selective feeding 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 gammarids' gut microbiome or immune system. In general, model predictions matched observed effects caused by waterborne exposure on gammarids' leaf consumption, energy processing and growth during short- and long-term assays, respectively. However, when complex horizontal (bacteria – aquatic fungi) and vertical (leaf-associated microorganisms – shredder)

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

1 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, shredders' secondary production 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 shredders' growth indirectly (Bundschuh et al., 2017; Konschak et al., 2020a). It is yet unknown how combined effects of these groups of antimicrobials affect decomposer-detritivore systems.

We here 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 comprised 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 gammarids' energy processing (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; Zubrod et al., 2015a; Konschak et al., 2020a; Konschak et al., 2020b). Thereby, we aim at stimulating future research ultimately supporting the 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 horizontal and vertical species interactions (e.g., Romaní et al., 2006).

2. Materials and methods

2.1 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). 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 gammarids' leaf consumption 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).

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., 2020a).

In January 2018, a 24-day long-term assay was conducted to assess effects on the gammarids' energy processing, 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.

2.2 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., 2020b) and CIP (98%, Acros Organics, Geel, Belgium; cf. Konschak et al., 2020a) 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 Fischer 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.

2.3 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 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.

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 test vessel's bottom. 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 gammarids' feces production (Zubrod et al., 2015b) and leaf disc remains were dried and weighed to determine gammarids' leaf consumption. 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.

2.4 Microbial and fatty acid 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 and Schmitt (1996). Furthermore, three leaf discs were used to determine bacterial density through fluorescence microscopy according to Buesing (2007). The community composition of aquatic hyphomycetes, a pivotal fungal group for microbial conditioning (Bärlocher, 1985), was analyzed through their spore morphology. To do so, five additional leaf discs per aquarium were shaken (120 rpm) for 96 h in deionized water at 16 ± 1°C. The community composition was analyzed according to Pascoal and Cássio (2004) and spores were identified by using various identification keys (e.g., Ingold, 1975).

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 via gas chromatography with flame ionization detector according to Fink (2013) and Konschak et al. (2020a), respectively.

2.5 Calculations and statistics

Leaf consumption (as 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 best fitting model (based on Akaike's information criterion) was used (Table S2).

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 – 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 – 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.

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 and non-parametric unpaired data consisting of two factorial predictors and two factor levels were evaluated using two-way analysis of variance (ANOVA) and rank-transformed two-way ANOVA, respectively. Parametric unpaired data containing one factorial predictor with two factor levels and at least three factor levels were evaluated via Student's *t*-test and one-way ANOVA followed by Dunnett's test, respectively. Non-parametric unpaired data 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 were analyzed via paired *t*-tests and Wilcoxon signed-rank tests, 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. 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" is abbreviated with "significant" throughout the entire study.

3 Results

The model of Cedergreen et al. (2005) was the best fit to the feeding activity data as it is capable of reflecting hormetic (i.e., stimulation at low concentrations) responses (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).

During the food choice assay, the gammarids did not significantly prefer 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. S1). Furthermore, AZO and CIP significantly reduced fungal biomass and bacterial densities, respectively, while both antimicrobials significantly affected fungal sporulation (Table S5). The IA model's 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 (difference of ~30% and ~50%; Fig. 3b).

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, S2; 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's 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, the IA model's predictions for leaf consumption and growth did not match the median observed effects (difference of ~20% and ~60%; Fig. 4) in the Combined treatment.

4 Discussion

4.1 Short-term waterborne effects

As expected, effect predictions of the IA model mirrored observed effects of the binary antimicrobial mixture on gammarids' leaf consumption, except for the lowest tested concentration (Fig. 2). The increased leaf consumption (~30%) 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 components had been applied individually (Zubrod et al., 2014; Konschak et al., 2020a). The stimulated leaf consumption 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*.

4.2 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's 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., 2020a). 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. This applies for both mixtures of 0.1 + 0.1 and 2.5 + 0.1 mg/L AZO + CIP. When only CIP was present at 0.1 mg/L, it stimulated microbial leaf decomposition (Konschak et al., 2020a), 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.

4.3 Long-term waterborne and dietary effects

In accordance with the results of the IA model's predictions, no effects to the binary antimicrobial mixture via waterborne exposure were observed in *G. fossarum* during the long-term assay (Fig. 4). While the IA model's predictions for leaf consumption and growth fell within the 95% confidence intervals of the median observed effects of gammarids in the Diet treatment, the deviation (by 10%) 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 (c.f. Konschak et al., 2020a), resulting in turn in an increased growth and NLFA content. Konschak et al. (2020a) suggested a stimulation of the gammarids' leaf consumption 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 gammarids' gut microbiome or the

immune system as both can have an impact on the animals' energy processing and behavior (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 gammarids' leaf consumption were overestimated by the IA model's 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). Thus, except for feces production, predictions of the IA model did not comply with the observed mixture effects when both pathways acted jointly. 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 amphipods' differential responses. 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 AZP 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 further studies targeting the underlying processes and mechanisms to help develop a more holistic picture of the risks associated with the unintended release of antimicrobials into surface waters.

5 Conclusion

We here demonstrate that microbial decomposers and shredders can show unexpected effect patterns in the presence of antimicrobial mixtures when data of the mixture's individual components 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 are 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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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)
Feeding	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
activity	Konschak et al. (2020a)	CIP	500	80.0 + 500
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
	Konschak et al. (2020a)	CIP	0.1; 2.5	
Long-term	Konschak et al. (2020b)	AZO	15.0	15.0 + 500.0
	Konschak et al. (2020a)	CIP	500.0	

Figure 1 Schematic overview of the three assay designs (1a, 1b and 1c; following Konschak et al., 2020a). 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).

Figure 2 Mean (with ± 95% CI) percentage effect on gammarids' leaf consumption (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.

Figure 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.

Figure 4 Median (with ± 95% CI) percentage effect (relative to the control) of gammarids' leaf consumption (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.

Fig. 1

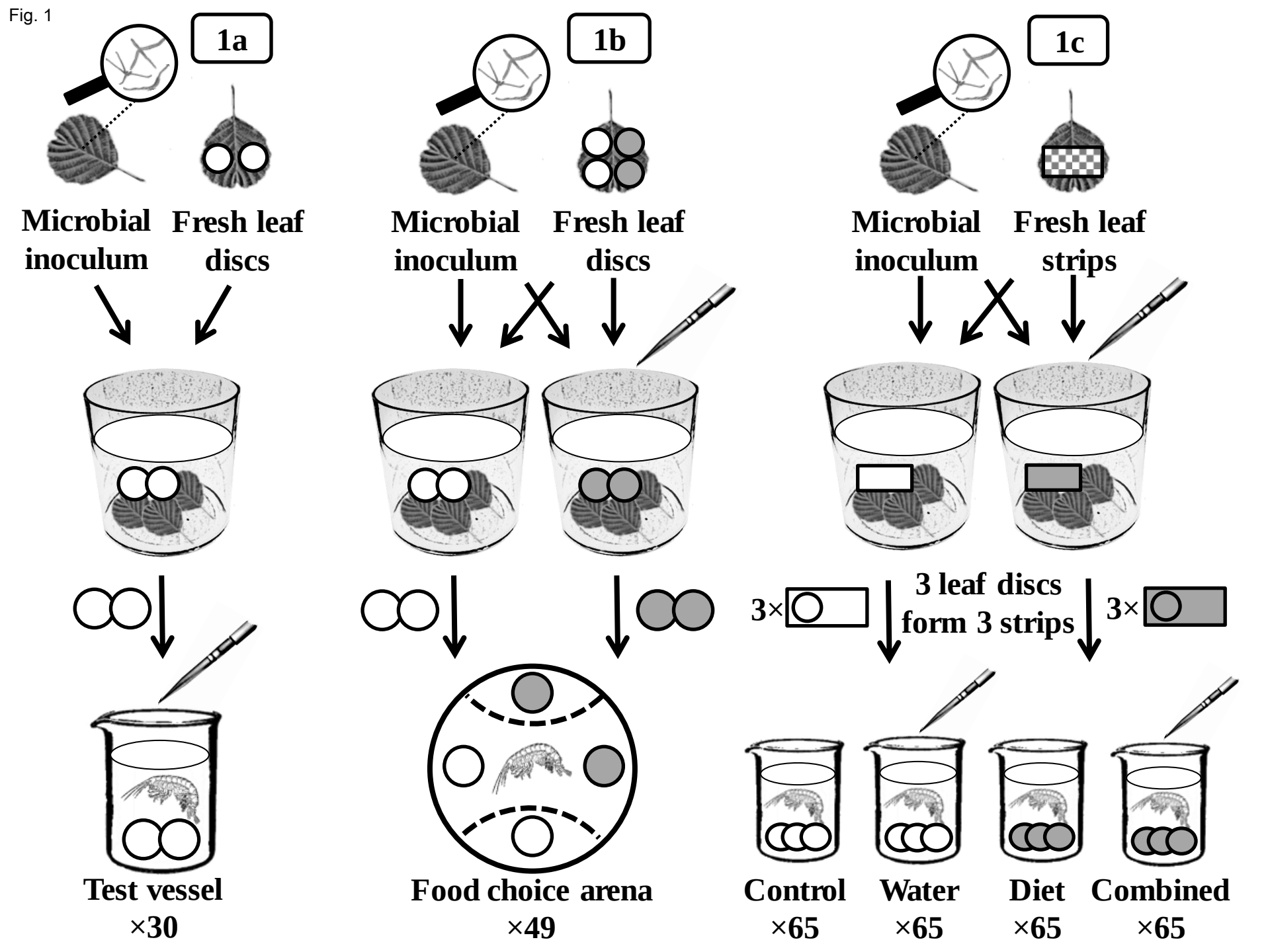

1a
1b
1c
Microbial inoculum
Fresh leaf discs
Microbial inoculum
Fresh leaf discs
Microbial inoculum
Fresh leaf strips
3×
3 leaf discs form 3 strips
3×
Test vessel
×30
Food choice arena
×49
Control
×65
Water
×65
Diet
×65
Combined
×65

Fig. 2

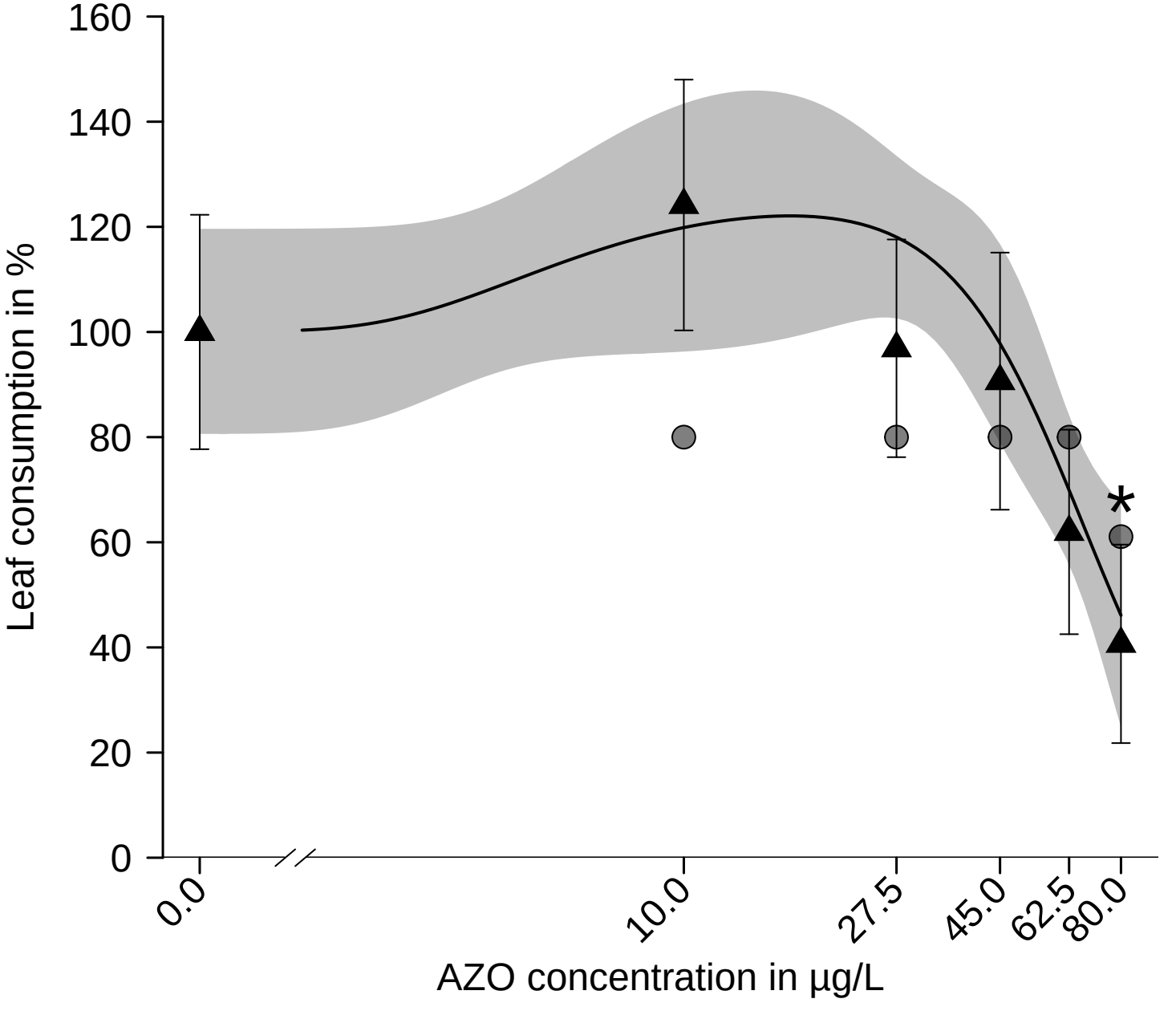

160
140
120
100
80
60
40
20
0
Leaf consumption in %
0.0
10.0
27.5
45.0
62.5
80.0
AZO concentration in µg/L
*

Fig. 3

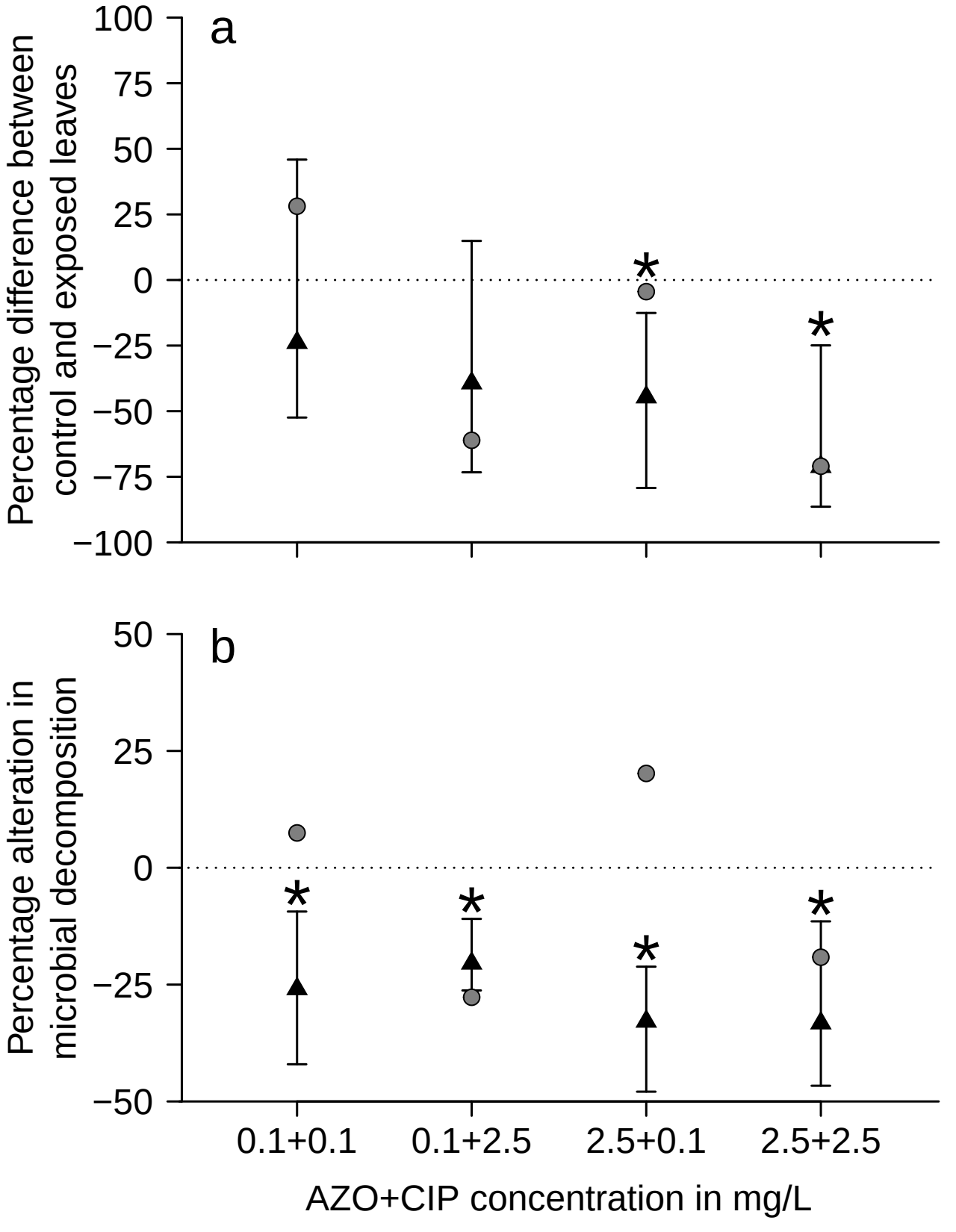
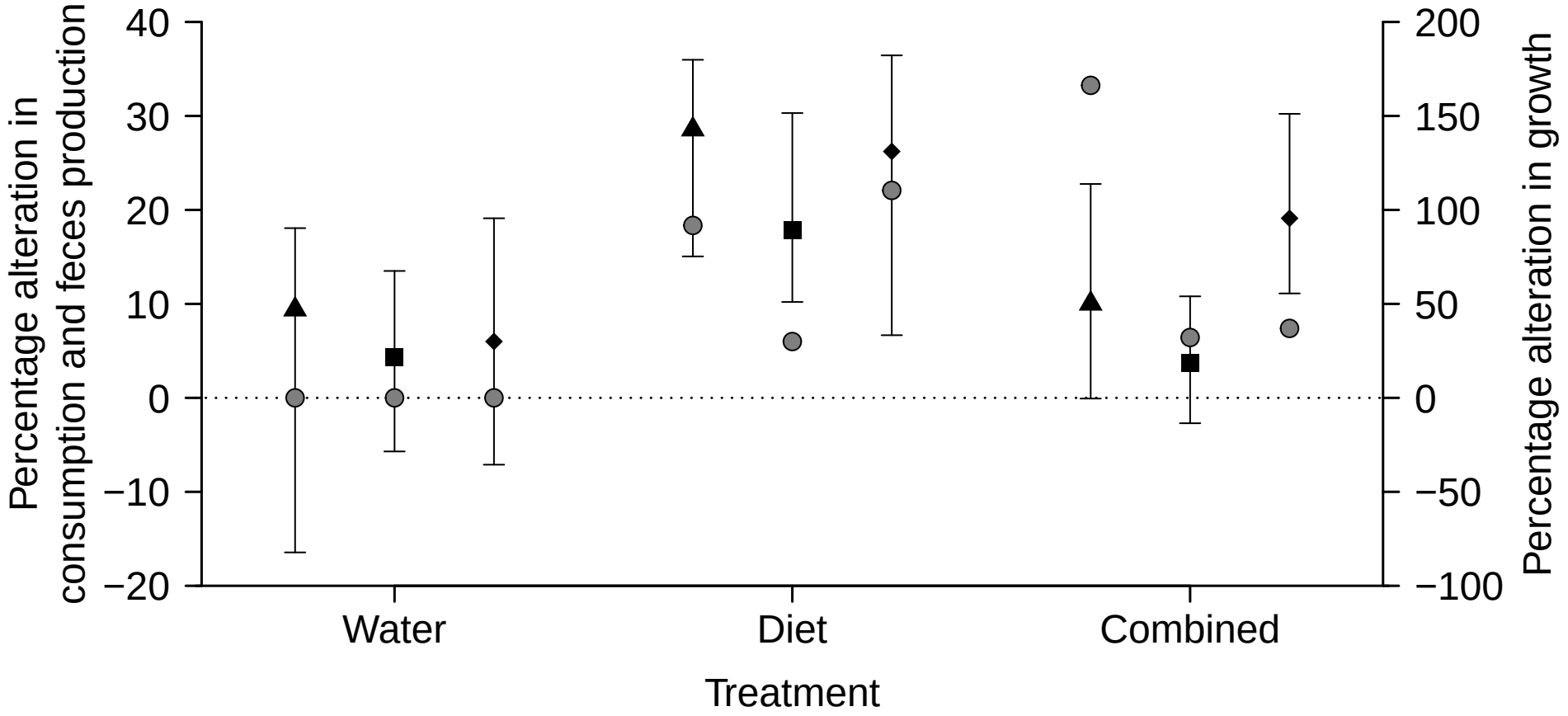

a
b
Percentage difference between control and exposed leaves
Percentage alteration in microbial decomposition
100
75
50
25
0
−25
−50
−75
−100
50
25
0
−25
−50
*
0.1+0.1
0.1+2.5
2.5+0.1
2.5+2.5
AZO+CIP concentration in mg/L

Fig. 4


Percentage alteration in consumption and feces production
40
30
20
10
0
−10
−20
200
150
100
50
0
−50
−100
Percentage alteration in growth
Water
Diet
Combined
Treatment