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

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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

1. 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 of triacylglycerols (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.

2. Materials and methods

2.1. 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 (Zubrod et al., 2014; . Bundschuh et al., 2009; 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 towards *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-hour 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. 1B3). 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.

2.2. 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 Fischer Sientific, 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.

2.3. 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 ~1 mm) 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 – 1.6 mm 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 hours before test start.

2.4. 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 hours, autoclaved (to avoid unintended indirect, diet-related effects on *G. fossarum*; model DE-65®, Systec, Linden, Germany), dried again for 24 hours 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 hours to reduce buoyancy.

The 7-days 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.

2.5. 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 hours at 60°C, weighed individually to the nearest 0.01 mg, and re-soaked with autoclaved SAM-5S for 48 hours. 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 hours 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 hours 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.

2.6. 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 fatty acid 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). 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.

2.7. 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 hours (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, 2007). Ergosterol was extracted via solid-phase extraction (Sep-Pak® Vac RC tC_{18} 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 (250 mm × 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 (2007). Briefly, bacterial cells were detached from the discs using ultrasonication and 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.

2.8. Fatty acid analyses

We quantified triacylglycerol (TAG) fatty acids (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).

2.9. 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-days 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 % of 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 means or medians with 95 % confidence intervals) of the assays are provided in Tables 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.

3. Results and discussion

3.1. 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 towards 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.

3.2. 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).

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.

3.3. 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,

~15 %; 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).

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 S6 and 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.

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*.

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.

3.4. 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 and thus at 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.

4. 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 exacerbate energy shortage from spring until autumn for the entire community.

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Figure 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).

Figure 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.

Figure 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)

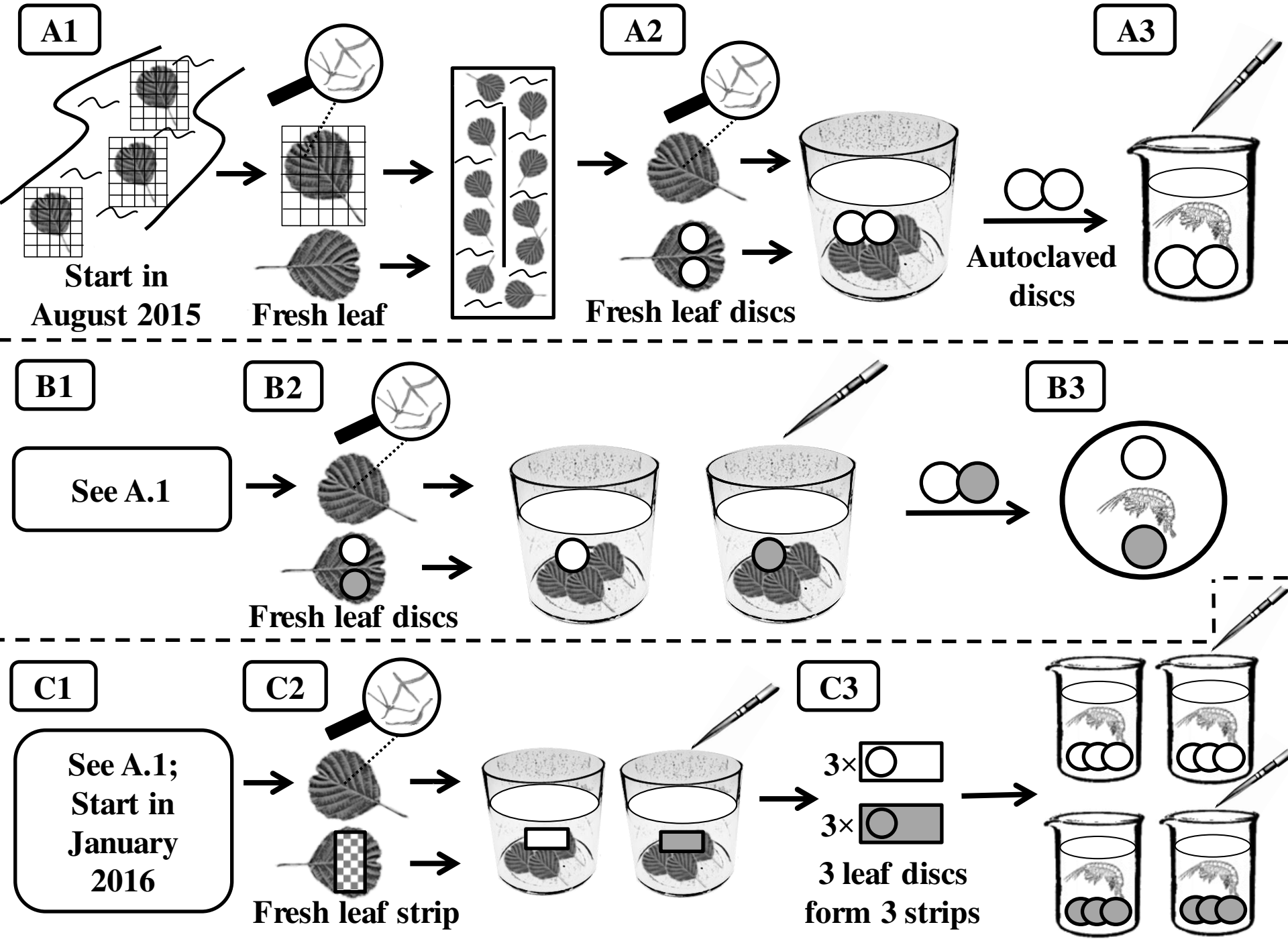
Figure 4 Mean relative leaf consumption (with 95% CIs) of *G. fossarum* on leaves microbially colonized under control conditions (white bars) or in the presence of increasing CIP concentrations (grey bars). The dotted line indicates the no-effect level (i.e., 50% consumption on both leaf types).

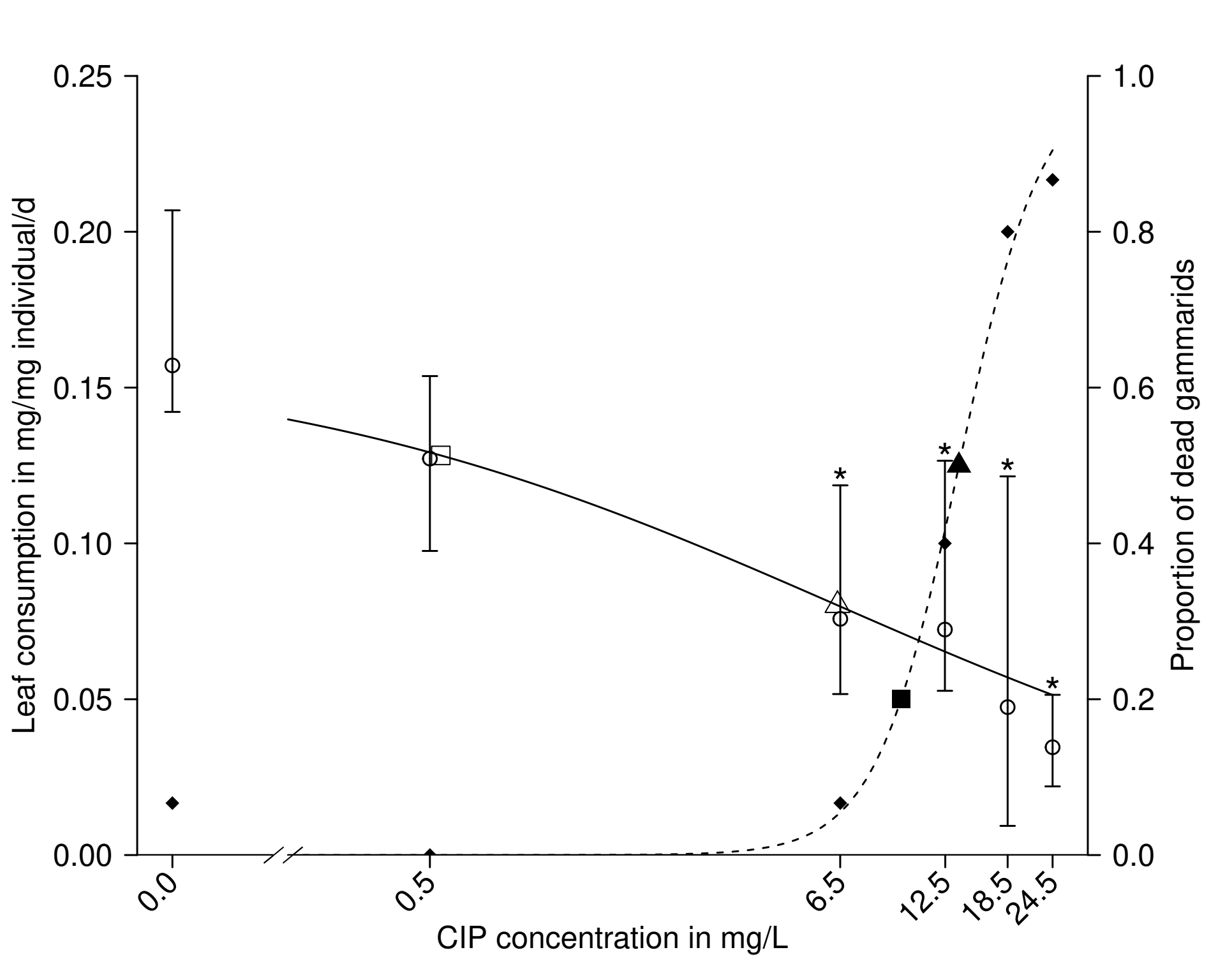
Figure 5 Median (with 95% CIs) leaf consumption (points), feces production (triangles) and growth (diamonds) of *G. fossarum* subjected to different effect pathways during the long-term feeding assay with CIP. Statistical analyses are displayed in Table 1.

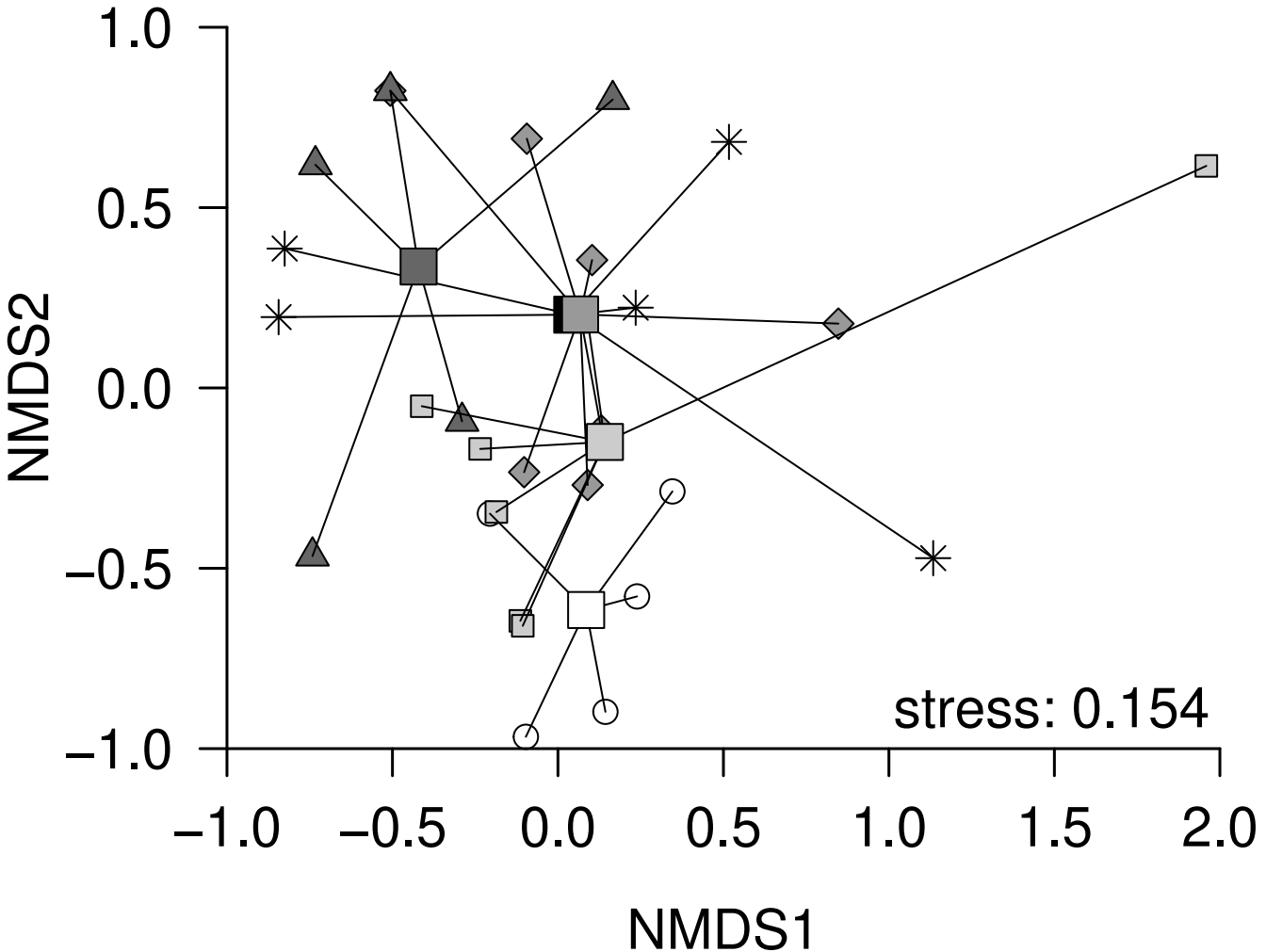
Figure 6 Median (with 95% CIs) saturated (SAFA; points), monosaturated (MUFA; triangles) and polysaturated (PUFA; diamonds) fatty acid content of *G. fossarum* subjected to different effect pathways during the long-term feeding assay with CIP. Statistical analyses are displayed in Table 1.

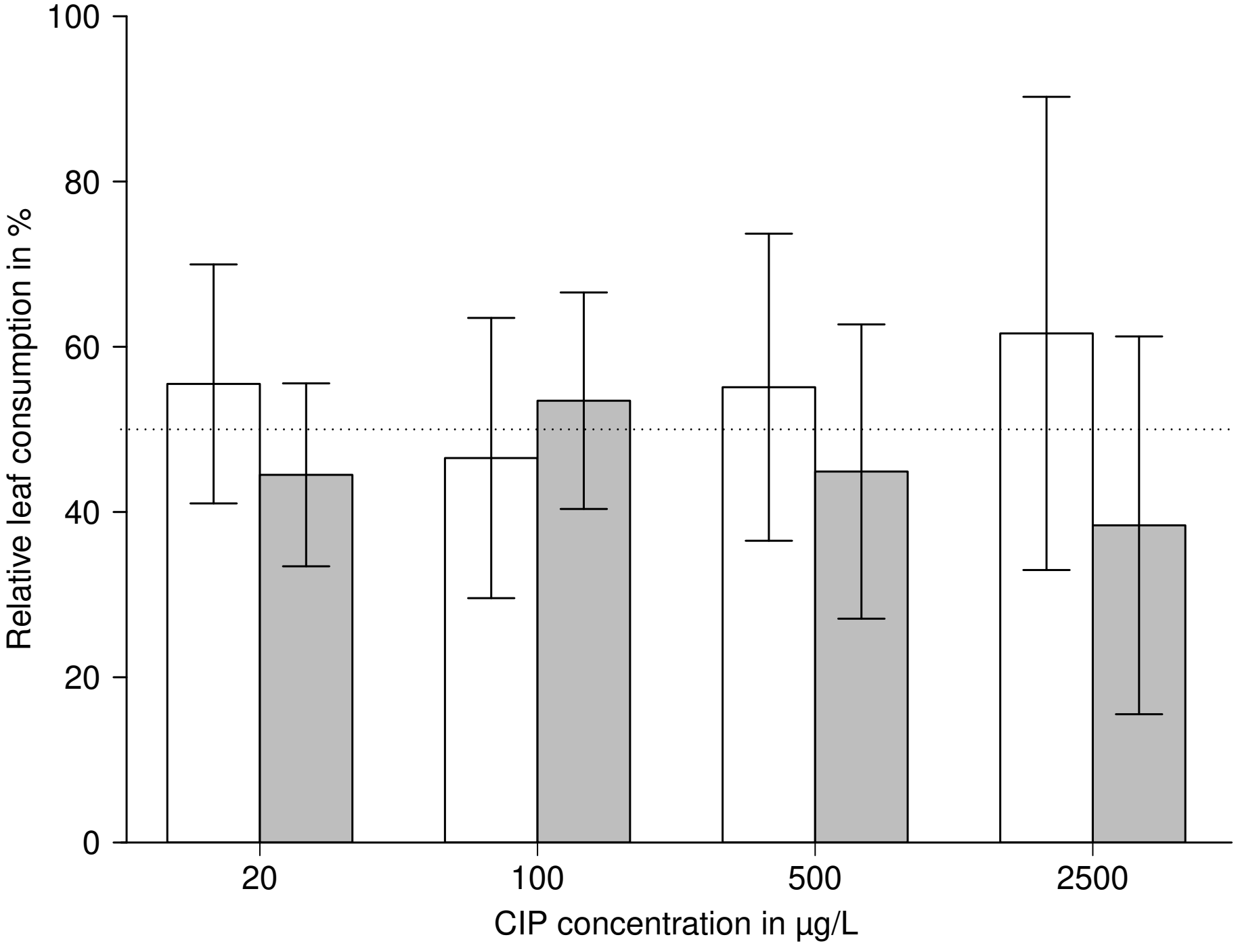
Table 1 ANOVA-tables for all gammarid-related endpoints during the long-term feeding assay. All *p*-values <0.05 are printed in bold.

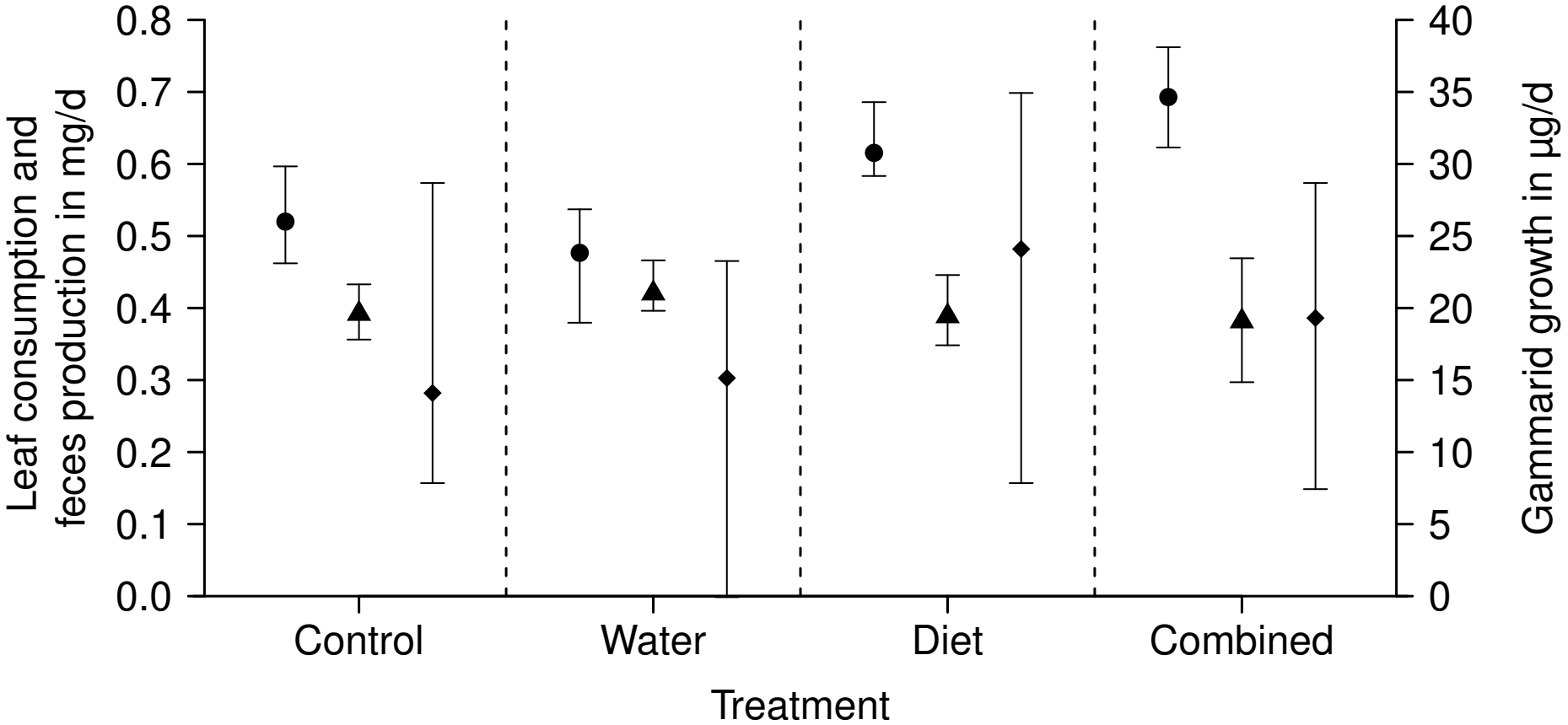
Endpoint	Factor	df1	SS/df2	MS/R2	*F*-value	*p*-value	ANOVA type
Leaf consumption	Water	1	0.016	0.0163	0.295	0.588	Two-way ANOVA
	Diet	1	1.478	1.4783	26.764	**<0.001**	
	Water × Diet	1	0.260	0.2598	4.703	**0.031**	
	Residuals	233	12.869	0.0552			
Feces production	Water	1	222.6602	-	0.187	0.666	Brunner-Dette-Munk 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	Rank transformed 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			

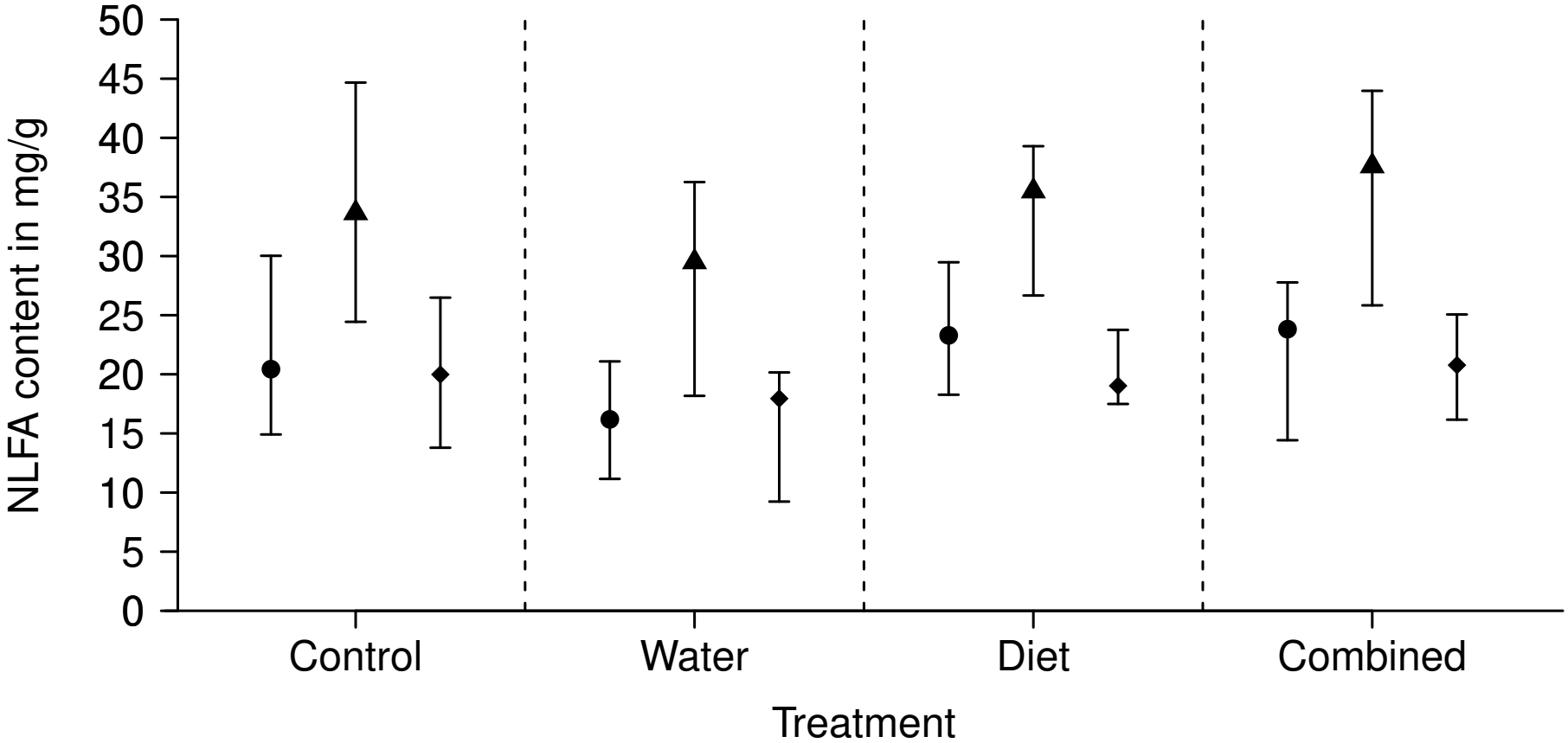

A1
Start in August 2015
Fresh leaf
A2
Fresh leaf discs
A3
Autoclaved discs
B1
See A.1
B2
Fresh leaf discs
B3
C1
See A.1; Start in January 2016
C2
Fresh leaf strip
C3
3×
3×
3 leaf discs form 3 strips


0.25
0.20
0.15
0.10
0.05
0.00
Leaf consumption in mg/mg individual/d
1.0
0.8
0.6
0.4
0.2
0.0
Proportion of dead gammarids
*
*
*
*
0.0
0.5
6.5
12.5
18.5
24.5
CIP concentration in mg/L


1.0
0.5
0.0
−0.5
−1.0
NMDS2
−1.0
−0.5
0.0
0.5
1.0
1.5
2.0
NMDS1
stress: 0.154


100
80
60
40
20
0
Relative leaf consumption in %
20
100
500
2500
CIP concentration in µg/L


Leaf consumption and feces production in mg/d
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Gammarid growth in µg/d
0
5
10
15
20
25
30
35
40
Control
Water
Diet
Combined
Treatment


50
45
40
35
30
25
20
15
10
5
0
NLFA content in mg/g
Control
Water
Diet
Combined
Treatment

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

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Preliminary feeding activity assay with *Gammarus fossarum*

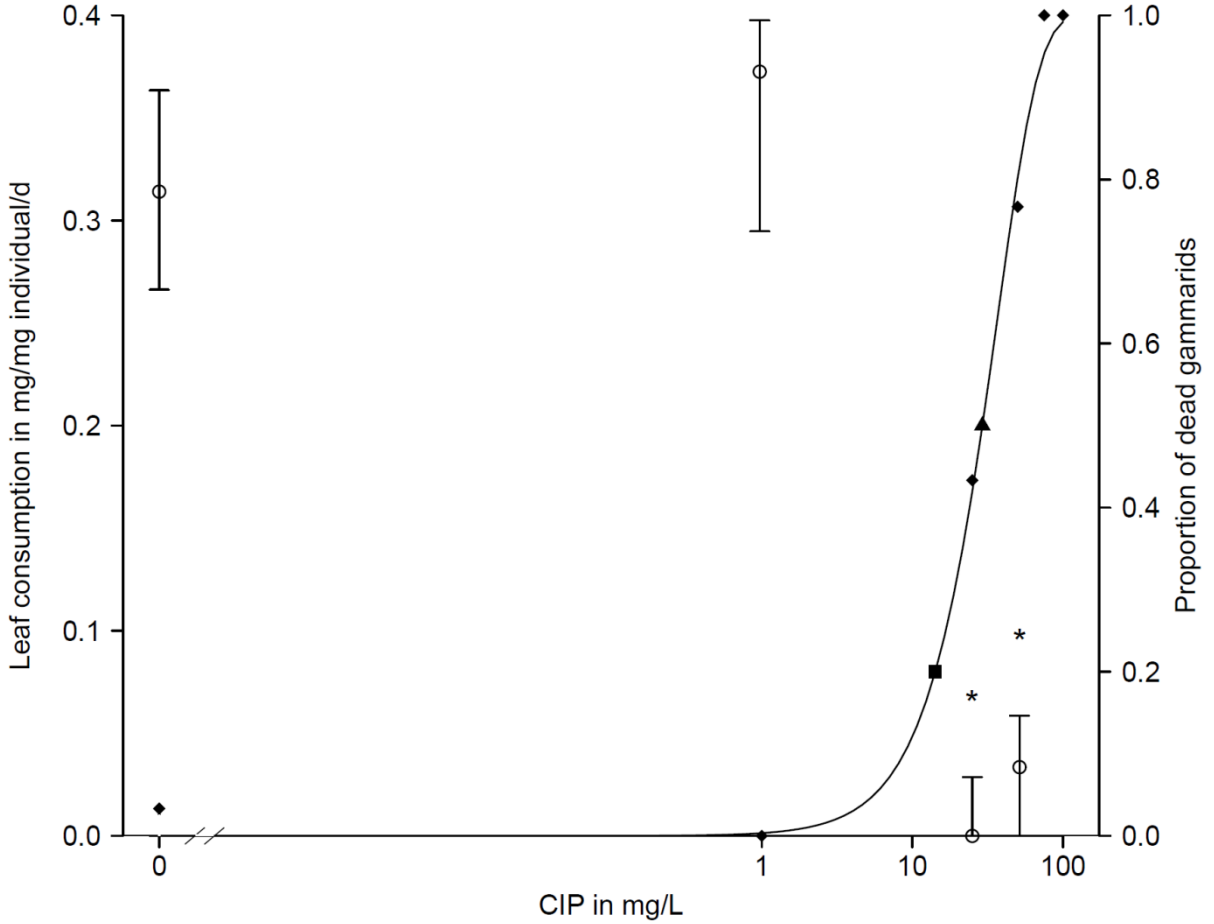

Figure S1 Median leaf consumption (open circles with 95% CIs; n = 30) of *G. fossarum* and proportion of dead gammarids (solid diamonds) when exposed to increasing ciprofloxacin (CIP) concentrations (0, 1, 25, 50, 75, 100 mg/L) for seven days. Moreover, the model with the best fit for mortality (see 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

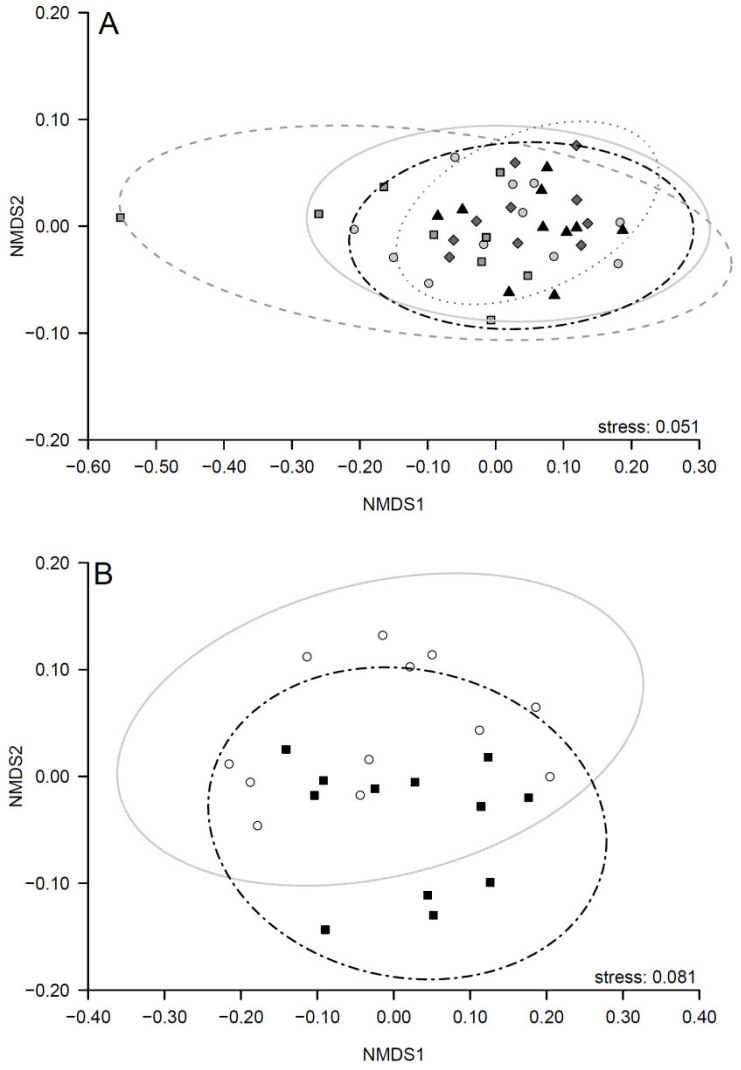

Figure S2 Non-metric multidimensional scaling (NMDS) plots for the NLFA composition of **A** gammarids subjected to four treatments during the long-term feeding assay with 500 µg CIP/L: a CIP-free control (i.e., Control; light grey circles), gammarids being directly exposed to CIP (i.e., Water; grey 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	Concen-tration (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
Mycocentro-spora 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	Concen-tration (µg/L)	Median	±95% CI	Statistical test	*p*-value
11:0	0	0.00	0.00 to 0.00	Wilcoxon rank-sum	0.106
	500	0.00	0.00 to 0.01		
12:0	0	0.03	0.03 to 0.05	Wilcoxon rank-sum	**0.017**
	500	0.02	0.02 to 0.04		
13:0	0	0.00	0.00 to 0.00	Student's t (unpaired)	0.935
	500	0.00	0.00 to 0.00		
14:0	0	0.09	0.08 to 0.11	Student's t (unpaired)	0.335
	500	0.08	0.06 to 0.11		
15:0	0	0.01	0.01 to 0.01	Wilcoxon rank-sum	0.242
	500	0.01	0.01 to 0.01		
16:0	0	1.10	0.64 to 1.45	Student's t (unpaired)	0.085
	500	0.76	0.59 to 1.19		
17:0	0	0.04	0.03 to 0.06	Student's t (unpaired)	0.156
	500	0.04	0.03 to 0.05		
18:0	0	0.12	0.07 to 0.13	Student's t (unpaired)	0.502
	500	0.11	0.09 to 0.16		
20:0	0	0.19	0.14 to 0.44	Wilcoxon rank-sum	0.078
	500	0.40	0.29 to 0.48		
21:0	0	0.02	0.01 to 0.02	Wilcoxon rank-sum	0.143
	500	0.02	0.02 to 0.03		

Table S9 continued.

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

References

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