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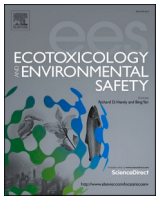

Direct and indirect effects of chemical pollution: Fungicides alter growth, feeding, and pigmentation of the freshwater detritivore *Asellus aquaticus*

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ABSTRACT

Anthropogenic chemical pollutants, such as fungicides, pose significant threats to natural ecosystems. Although the direct impacts of numerous chemicals are well-documented in simple environmental contexts, their indirect impacts are poorly understood. This study used two individual level laboratory experiments to assess direct and indirect effects of fungicides on the isopod *Asellus aquaticus*, a keystone detritivore in freshwater systems. First, a range-finding assay on three widely used fungicides (Fluazinam, Tebuconazole, Urea) showed that Tebuconazole had the strongest concentration-dependent negative effects on *A. aquaticus* growth and food consumption. Second, a factorial experiment using Tebuconazole assessed its direct and diet-mediated effects and showed that Tebuconazole reduced growth, feeding, and pigmentation through both pathways. The results indicate that assessing only direct impacts of toxic chemicals could overlook critical interactions that are relevant in natural systems, such as those associated with diet. Our study highlights the importance of considering both direct and indirect effects in environmental toxicology to better understand the full impacts of chemical pollutants in nature.

1. Introduction

Human-induced chemical pollution is a major driver of global biodiversity loss (Groh et al., 2022), affecting ecosystems at levels ranging from cellular processes to food web dynamics and ecosystem processes (Stamm et al., 2016). One group of chemicals widely used and frequently entering natural ecosystems is fungicides (Zubrod et al., 2019). While fungicides are designed to control harmful fungi, they are also known to affect general biological processes such as energy production of non-target organisms (Maltby et al., 2009) and indirectly alter food web dynamics by influencing the resource base of consumers.

Despite agricultural benefits (Zubrod et al., 2019), fungicides leach into aquatic ecosystems, impacting non-target fungi and associated taxa (Tofan et al., 2023). Understanding the impacts of fungicides on aquatic ecosystems, which harbor significant biological diversity while heavily affected by anthropogenic activities (Reid et al., 2019), is crucial because they can directly disrupt cellular processes (e.g. energy production, cell division, and nucleic acid synthesis; Stenersen, 2004), and also have potential indirect effects. The direct impacts of fungicides have been studied in a range of non-target species such as fishes, amphibians, and aquatic invertebrates (Wang et al., 2021, Hopkins and Hoverman, 2023). However, indirect effects of fungicides are little studied to date. Notably, some fungicides can persist in the environment for a long time (Hakala et al., 2020) and, beyond direct toxicity, harm the food web by altering fungal biomass and diversity, affecting detritivores that depend on fungi both as a nutritional resource and for making the leaf litter more palatable (Feckler et al., 2016).

This study focuses on the keystone detritivore, *Asellus aquaticus*, which is common in detritus-based food webs of freshwater ecosystems. *A. aquaticus* influences nutrient cycling and energy transfer, relying on fungi for nutrition (Marcus and Willoughby, 1978, Graça et al., 1993) and break-down of the complex plant cell walls in the leaf litter (Tennakoon et al., 2021), thereby facilitating its dietary intake and promoting survival and growth (Rossi and Fano, 1979). Past studies suggest that *A. aquaticus* prefers food with higher fungal biomass and certain fungal taxa over others (Graça et al., 1994, Bundschuh et al., 2011). *A. aquaticus* is also an important prey species for invertebrates, and fish, and an intermediate host for fish parasites (Lafuente et al., 2021), indicating that potential effects of fungicides on *A. aquaticus* could transfer across different trophic levels.

In addition to the effects of direct fungicide exposure, we also explore how fungicides, via indirect pathways such as alterations in diet quality,

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impact the growth, feeding behavior, and pigmentation of *A. aquaticus*. Changes in these traits, for instance, pigmentation, may lead to increased predation rates of *A. aquaticus* and decreased protection from UV exposure down the line (although these parameters were not evaluated in the present study), and subsequently impact nutrient cycling within the ecosystem.

We first assess the sensitivity of *A. aquaticus* to three commonly used fungicides (Fluazinam, Tebuconazole, Urea) in a range finding assay, and then assessed direct and indirect (diet-mediated) effects of Tebuconazole in a 4 ×4 factorial experiment, which showed clear toxic effects in the range-finding assay. Tebuconazole has a multi-targeted mode of action on fungi (Shikuku et al., 2014) by suppressing fungal spore germination and growth and interfering with ergosterol production (component of fungal cell walls). Tebuconazole is hazardous to both terrestrial and aquatic life (Yao et al., 2022, Zubrod et al., 2011), and is commonly used in Europe and has been detected in surface waters at concentrations ranging from 9.1 μg/L (Berenzen et al., 2005) up to 200 μg/L (Elsaesser and Schulz, 2008). Because of its toxicity and potential to influence dietary resource quality of *A. aquaticus*, we hypothesized that even at environmentally relevant concentrations, Tebuconazole may affect *A. aquaticus* performance – which could have wider consequences for the functioning of freshwater ecosystems. We assessed *A. aquaticus* responses to fungicides through survival, growth, pigmentation and feeding efficiency, which encompass traits relevant for performance and ecological function of the species (Lafuente et al., 2021, Lürig et al., 2019). We predicted that at harmful concentrations, *A. aquaticus* survival and/or growth would be reduced in a concentration dependent manner. Furthermore, the effects of the indirect exposure pathway (diet-mediated effects) should be visible as decreases in growth and/or food consumption due to lower nutritional value of the food source (leaf litter). Jointly, the direct and indirect effects of Tebuconazole should manifest as relatively stronger effects when both the individual and its food source are affected.

2. Materials and methods

2.1. Study species and population

We sourced juvenile *A. aquaticus* from lake Konnevesi (62° 38′ N and 26° 24′ E) in central Finland, a medium-sized lake (around 187 km^2 in size) with circumneutral pH and excellent water quality (Noori et al., 2022). A mix of adults and juveniles (N=540) picked randomly from the lab stock (collected in September 2021), were used for the range-finding assay. For the direct-indirect effects experiment, approximately 350 juveniles (2–4 mm) were collected in June 2022 using kick nets near the Konnevesi Research Station, sorted out from plant material with soft forceps and cut plastic pipettes, and then transported to the Ambiotica laboratory at the University of Jyväskylä.

Here, they were maintained in walk-in climate rooms and acclimatized in glass containers (20 ×20 x 30 cm tanks) under controlled conditions (18°C, 18:6 hours light:dark cycle) for at least a week and fed abscised black alder leaves (*Alnus glutinosa*), which makes a prominent part of the littoral leaf litter in many lakes in Finland. Alder leaves are soft, rich in nitrogen, and have high microbial activity (Caseiro et al., 2000), and hence make a good quality diet for isopods. Individuals for the Direct-Indirect effects experiment were picked randomly from the wild collected sample.

2.2. Chemicals used

Urea, Fluazinam, and Tebuconazole, chosen for their varied action modes and relevance in Europe, were tested (Table A.1). The commercially available product Shirlan (Product code: HM80052, manufactured by Syngenta Crop Protection AG) was used for testing the effects of Fluazinam and Folicur Xpert (Product code: HM16264, manufactured by Bayer) for Tebuconazole. The active ingredient in Shirlan is 500 g/L Fluazinam and Folicur Xpert contains 160 g/L of Tebuconazole and 80 g/L Prothioconazole, which is also a systemic triazole fungicide. ACS reagent (U5128, Sigma Aldrich) was used for testing the effects of Urea. For each of these chemicals, stock solutions (100 mg/L for Urea and 5 g/L for Fluazinam and Tebuconazole) were prepared with further serial dilutions for appropriate concentrations for the experiments. It is important to note that while the primary focus was on adjusting Tebuconazole's concentration, this dilution process also resulted in the proportional presence of Prothioconazole in our solution, at a concentration of 2.5 g/L, given its original ratio in Folicur Xpert. Though the fungicide concentrations during the exposure periods were not measured, stock solutions were prepared immediately before experiments in animal-safe ground water to ensure consistency. The solution within the well plates were changed once a week, and there was negligible evaporation, so it was assumed that the concentrations during the exposure periods remained consistent. Fluazinam has a moderate degradation rate in aquatic systems with a half-life of 42 days at pH 7 and 25°C and forms a primary metabolite CAPA which retains the structural features of the parent compound (U.S. Environmental Protection Agency, 2001). Tebuconazole is stable at pH 7 and 25°C, with a degradation half-life in water of 198 days, and no major metabolites (European Chemicals Agency, 2023). Urea is highly soluble in water and undergoes rapid hydrolysis to ammonia and carbon dioxide. In aquatic environments, the half-life of urea is typically less than 10 days (Urbańczyk et al., 2016). However, it is the hydrolysis of urea is what gives it the fungicidal properties (Table A.1).

2.3. Experimental design

The study comprised a range-finding assay and a factorial experiment to explore direct and indirect fungicide effects on *A. aquaticus*, since the fungicides used were expected to affect either or both *A. aquaticus* themselves or the fungal communities on leaf litter they consume.

2.3.1. Range-finding assay

In the range-finding assay, individual *A. aquaticus* were exposed to one of three fungicide treatments (Urea, Fluazinam, Tebuconazole), across 10 different concentrations, with 18 replicates per treatment (N = 540 *A. aquaticus*). For each treatment concentration, the 18 replicates (individual isopods) were assigned randomly across three 6-well plates. The experiment lasted for 21 days and was conducted in a climate chamber (HiPoint EH-1800 Plant Growth Chamber) at 18°C and an 18:6 hours light:dark cycle, with 150 lux light intensity. These isopods were fed standardized leaf discs prepared as per Protocol A.1. We chose a 21-day exposure period partially based on previous studies that have demonstrated significant effects on growth and feeding within this timeframe or less (Van Ginneken et al., 2018). Moreover, *A. aquaticus* molts every 7–10 days, and growth occurs only after molting and shedding the previous exoskeleton. During a 21-day period, there would be 3–4 moltings and hence potential to see growth responses.

Fungicide concentrations were chosen based on environmentally relevant scenarios, ranging from no traces of fungicides to environmentally relevant concentrations, to worst-case exposure scenarios (which were considered to be a 100-fold increase from the intermediate level). Such cases can occur, for example, when there is an extreme rainfall event soon after fungicide application and the subsequent leaching into water bodies increases the fungicide concentrations drastically. For urea, the 10 nominal concentrations were 0 mg/L, 0.25 mg/L, 0.5 mg/L, 1 mg/L, 2.5 mg/L, 5 mg/L, 10 mg/L, 25 mg/L, 50 mg/L, 100 mg/L, since the levels found in natural systems was around 1 mg/L (Finlay et al., 2010). For both Fluazinam and Tebuconazole, the 10 nominal concentrations were 0 μg/L, 2.5 μg/L, 5 μg/L, 10 μg/L, 25 μg/L, 50 μg/L, 100 μg/L, 250 μg/L, 500 μg/L and 1000 μg/L for because for both of these fungicides the environmentally relevant concentrations fall around 50 μg/L (Wang et al., 2018, Baudy-Groh et al., 2021).

2.3.2. Direct and indirect effects of Tebuconazole

To test the direct and indirect effects of Tebuconazole mediated via direct exposure of *A. aquaticus* versus treatment of food source, a fully factorial 4 × 4 experiment was conducted by exposing leaf discs and individual *A. aquaticus* to four different concentrations of Tebuconazole. Standardized leaf discs (diameter: 10 mm, prepared according to Protocol A.1) were pre-treated with four Tebuconazole concentrations (D0 = 0 µg/L, D1 = 5 µg/L, D2 = 50 µg/L, D3 = 500 µg/L) and subsequently used to feed *A. aquaticus* reared in corresponding Tebuconazole concentrations (T0 = 0 µg/L, T1 = 5 µg/L, T2 = 50 µg/L, T3 = 500 µg/L). This setup yielded 16 distinct treatment combinations (D0xT0, D0xT1, …, D3xT3).

Each of the 16 treatment combinations was replicated 18 times, with individual *A. aquaticus* reared in 6-well plates, resulting in a total of 288 individuals. The experiment ran for 35 days in summer conditions and was conducted in the same climate chambers as the range-finding assay. In the climate chamber there were 4 shelves, and the well plates were assigned across the shelves randomly and their position was changed daily.

Due to high mortality (10 out of 18) from a technical issue in the treatment group receiving a 50 µg/L diet with 0 µg/L exposure, we excluded all groups involving a 50 µg/L diet from the analysis. A follow-up experiment, conducted 10 months later with the same treatment configurations (under similar conditions using *A. aquaticus* from laboratory stock), confirmed the initial experiment's trends.

2.4. Response variables

Isopod growth, feeding, and pigmentation are expected to reflect toxic effects on isopod performance. Growth is a generic life-history trait relevant for individual performance. Feeding efficiency of individual isopods was measured both as a stress response, as well as a measure of how ecosystem function might be affected since *A. aquaticus* plays a major role in nutrient cycling in freshwater ecosystems (Lafuente et al., 2021). Pigmentation was measured because in *A. aquaticus*, it is known to be affected by its diet quality (Lürig et al., 2019), and because changes in pigmentation could affect interactions these isopods have with other trophic levels, such as predators (Lafuente et al., 2021).

Survival and molting in *A. aquaticus* were tracked daily by checking for deceased individuals and shed exoskeletons in their respective wells. Growth and pigmentation changes were evaluated through weekly digital photographs taken with a Canon EOS 850D equipped with a macro lens. Each isopod was provided with a single leaf disc throughout the experiment, with initial and final photographs of these discs serving to assess food consumption over a 3-week period.

For photographic documentation, each isopod was placed in an individual petri-plate against a white background, accompanied by a color and millimeter scale to ensure precise image analysis. Preliminary observations revealed that many isopods began to decrease in size after four weeks, likely due to constrained resources or less-than-ideal rearing conditions. Consequently, growth rate calculations were confined to the first 3 weeks of the experiment, during which isopods predominantly exhibited growth. Body size measurements were extracted from the digital images, quantifying area in square millimeters to an accuracy of 10^{-9} mm^2. The area included isopod body but removed appendages (Lürig et al., 2019). Growth rates were determined by calculating the difference between the initial and final body sizes, then dividing by the experiment's duration (21 days). Pigmentation analysis involved measuring grayscale values (ranging from 0 to 255, where 0 denotes no color and 255 the maximum intensity) across the isopod's body. The mean color value for gray was measured across the entire isopod and this value was further divided by the body size. These values were normalized using the formula (1-(mean gray value/255)), and pigmentation rates were calculated by comparing initial and final values over the 21-day period. Phenopype v3.3.4 (Lürig 2021), a Python-based software, facilitated the extraction of body size and pigmentation data. Feeding rates were evaluated by measuring the consumed area of the leaf discs, with Fiji software v1.54d (Abramoff et al., 2004) from the digital images before and after the experiment.

2.5. Statistical analyses

Data visualization and statistics were performed using R version 4.2.1 (R Core Team, 2022). We applied log-transformation to response variables to achieve normality and analyzed the effects using linear mixed models ('lmer' function, 'lme4' package). For each fungicide in the range-finding assay, concentration served as a fixed effect, with initial body size and its interactions as covariates, accounting for size-dependent effects on growth and feeding rates. In the direct-indirect effects experiment, we analyzed log-transformed growth, feeding, and pigmentation data using similar linear mixed models. These models incorporated Exposure (4 levels) and Diet (3 levels) as fixed factors, with initial size and its interactions as covariates to capture size-dependence of response variables (Vilisics et al., 2012). Plate identity was included as a random effect in all analyses to account for non-independence of individuals on the same plate. Model assumptions were tested through statistical tests and diagnostic plots, ensuring normality of residuals (Shapiro-Wilk test, Q-Q plots) and homogeneity of variance (residuals vs. fitted values plot, Levene's test). To assess the potential linear, quadratic, and higher-order trends across the concentrations in the range-finding models, we also performed polynomial contrasts. Exclusions for mortality were made for the individuals that died during the 3-week period for both sets of analyses. Survival (assigned a value of 0 or 1 depending on whether the individual was alive at the end of the experiment) was analyzed using the 'glmer' function in the 'lme4' package in R with a binomial error and a logit link function, considering Diet, Exposure, Initial size, and their interactions, with Plate identity as a random effect.

3. Results

3.1. Range-finding assay

Tebuconazole concentration affected growth and feeding rates, and Urea concentration had size-dependent effect on feeding rates of *A. aquaticus,* while Fluazinam had no significant overall concentration

Table 1
ANOVA Results from Linear Mixed Effects Models on Range-finding assays assessing the effects of three fungicides on growth and feeding rate of *A. aquaticus.* The full models included Fungicide concentration, and Initial size interactions. All models included Plate number as a random effect. Only final models are presented. Significant effects are highlighted in bold.

	Fluazinam		*Tebuconazole*		*Urea*	
Predictor	$F_{ndf,ddf}$	*P*	$F_{ndf,ddf}$	*P*	$F_{ndf,ddf}$	*P*
Growth rate						
Concentration	$F_{9,152} = 1.56$	0.130	$\mathbf{F_{9,152} = 2.42}$	**0.013**	$F_{9,30} = 1.37$	0.242
Feeding rate						
Concentration	$F_{9,171} = 0.75$	0.659	$\mathbf{F_{9,33} = 2.16}$	**0.051**	$F_{9,159} = 1.67$	0.097
Initial size	$F_{1,171} = 4.90$	**0.028**	$F_{1,161} = 67.67$	**<0.001**	$F_{1,159} = 45.03$	**<0.001**
Concentration * Initial size	–	–	–	–	$\mathbf{F_{9,159} = 2.03}$	**0.039**

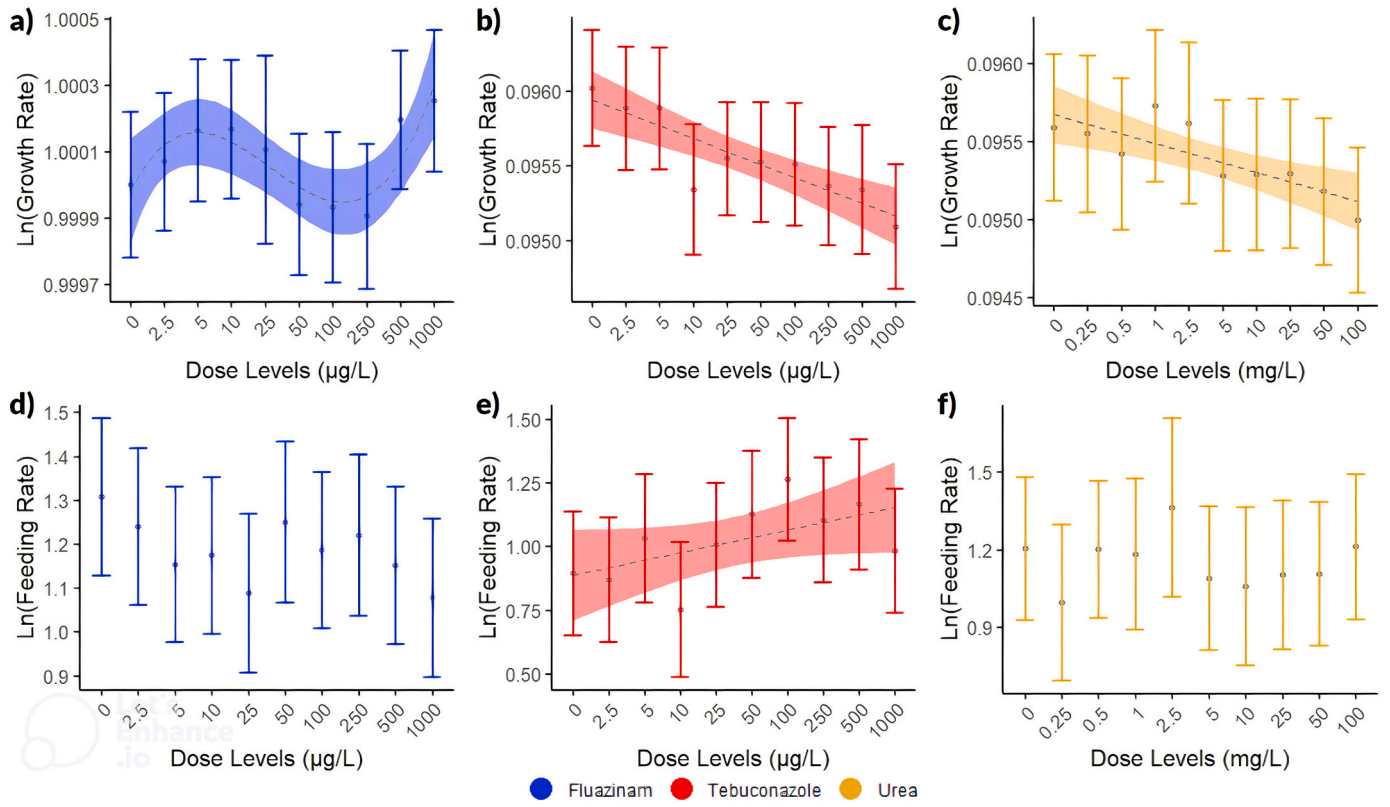

Fig. 1. Polynomial trends in growth (panels a, b, c) and feeding rates (panels d, e, f) at various concentrations of Fluazinam, Tebuconazole, and Urea. The predicted mean values from the respective linear mixed models are overlaid on the trend lines (only the significant polynomial trends are shown here), with shaded areas indicating the 95 % confidence intervals for the estimates.

dependent effects on either variable (Table 1). In general, growth rates were unaffected by initial size, but individuals that were larger at the start of the experiment had faster food consumption rates than smaller individuals (Table 1, Fig.A.1). In some urea treatments, however, larger and smaller individuals had similar growth rates (Fig.A.2).

While there was no overall concentration level effect as a fixed factor for Fluazinam, tests of polynomial trends found a cubic trend on *A. aquaticus* growth rate (Estimate = 0.05 ± 0.009, $P = 0.01$; Fig. 1), whereby growth rates first increased with increasing concentration (0–5 µg/L) but decreased in the mid-high concentrations (50–250 µg/L), and then increased again (250–1000 µg/L). Growth rates decreased (Estimate = −0.01 ± 0.003, $P = 0.0006$), but intriguingly feeding rates increased linearly (Estimate = 4.89 ± 2.23, $P = 0.03$; Fig. 1) with increasing Tebuconazole concentrations. Growth rates decreased linearly with increasing concentrations of urea (Estimate = −0.01 ± 0.004, $P = 0.02$; Fig. 1).

3.2. Direct and indirect effects of Tebuconazole

3.2.1. Survival and molting

37 of the 216 (17 %) experimental individuals died over the course of the Direct-Indirect experiment, and 144 (66 %) molted at least once. Survival ($\chi^2 = 5.32$, $df = 1$, $P = 0.02$), was significantly affected by initial body size, but there were no significant treatment effects, or treatment-initial size interactions on either response variable.

3.2.2. Growth rates

While direct Exposure had no significant effects on *A. aquaticus* growth rates (Table 1), Diet had significant size-dependent effects (Diet * Initial size, $P = 0.04$; Table 2): while there was a positive relationship between initial size and growth in the 0 µg/L diet treatment (beta = 0.192 ± 0.05, $P = 0.0002$; Fig. 2), there was no significant relationship between initial size and growth in the 5 µg/L and 500 µg/L diet treatments (Fig. 2, Table B.1). Specifically, smaller isopods (< 2 mm^2) showed similar growth in all diet treatments, but larger individuals had slower growth rates in 5 µg/L and 500 µg/L diets compared to those that

Table 2
ANOVA Results from Linear Mixed Effects Models on effects of Tebuconazole on growth rates, feeding rates, and pigmentation change of *A. aquaticus*. The full models included Exposure, Diet, and Initial body size interactions. All models included Plate number as random effect. Only final models are presented. Significant effects are highlighted in bold.

	Growth rate		*Feeding rate*		*Pigmentation change*	
Predictor	$F_{ndf,ddf}$	*P*	$F_{ndf,ddf}$	*P*	$F_{ndf,ddf}$	*P*
Exposure	$F_{3,191} = 1.31$	0.269	**$F_{3,36} = 9.43$**	**<0.001**	**$F_{3,191} = 5.57$**	**0.001**
Diet	$F_{2,191} = 0.21$	0.810	**$F_{2,36} = 6.01$**	**0.005**	$F_{2,191} = 1.49$	0.226
Initial size	**$F_{1,191} = 6.40$**	**0.012**	**$F_{1,181} = 11.21$**	**<0.001**	**$F_{1,191} = 11.39$**	**<0.001**
Exposure * Diet	$F_{6,191} = 0.29$	0.940	$F_{6,36} = 0.37$	0.890	**$F_{6,191} = 3.17$**	**0.005**
Diet * Initial size	**$F_{2,191} = 3.07$**	**0.048**	–	–	–	**–**

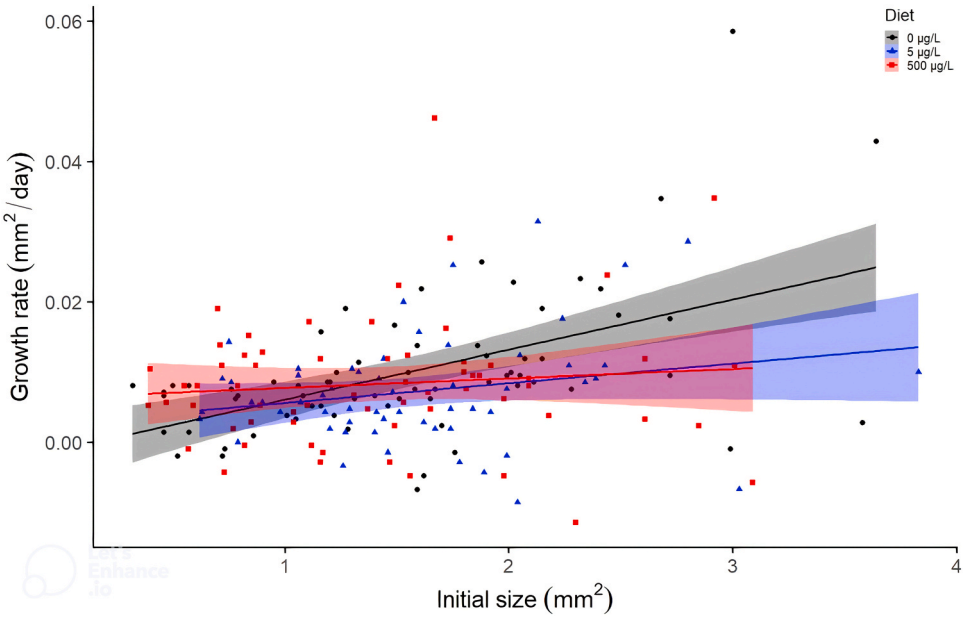

Fig. 2. Linear regression lines showing the relationship between initial size (mm^2) and growth rate (mm^2/day) of *A. aquaticus* that consumed leaf discs treated with different Tebuconazole concentrations (Diet treatments). Raw data alongside the respective 95 % confidence intervals is plotted here, with each point presenting the values for individual *A. aquaticus*.

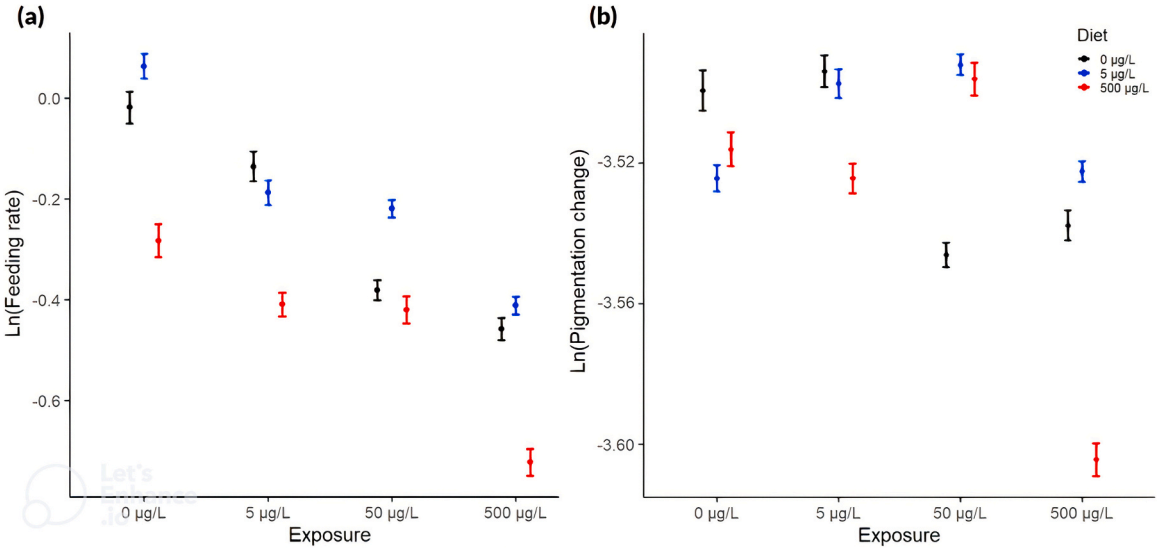

Fig. 3. Least square means ± Standard errors of (a) ln(feeding rates) and (b) ln(pigmentation change), predicted from linear mixed effects models (Table 2) across different exposure concentrations of direct (Exposure: 0, 5, 50, and 500 µg/L) and indirect (Diet: 0, 5, and 500 µg/L) effects. Bigger values for (a) indicate that individuals ate more, and for (b) indicate that for a given size, individuals became darker.

fed on the 0 µg/L diet indicating that larger isopods suffer decreased growth when their diets were exposed to fungicide.

3.2.3. Feeding rates

Increased levels of Tebuconazole reduced *A. aquaticus* feeding rates through both Exposure and Diet treatments (Fig. 3a, Table 2), but there was no significant Exposure * Diet interaction indicating that the effects were additive. The effects of Exposure (direct effect) were stronger than Diet-mediated (indirect) effects (Table 2). Larger isopods had higher feeding rates but there was no size dependence in treatment effects (Table 2). Pairwise comparisons of least square means revealed that for any given diet level, feeding rates declined on average by ~34–36 % through direct exposure and by ~21–25 % through diet-mediated effects when comparing the 0 µg/L and 500 µg/L treatments (Fig. 3a, Table B.2).

3.2.4. Pigmentation change

In contrast to growth and feeding rates, pigmentation change showed interactive effects between Exposure and Diet (Exposure * Diet: $P = 0.005$, Table 2, Fig. 3b) as well as a significant Exposure main effect ($P = 0.001$, Table 2).

A significant effect of initial body size indicated that larger individuals changed their pigmentation more (became darker) in all treatments ($ß = 0.0354 \pm 0.011$, $P = 0.0015$). The pigmentation change seemed to remain consistent for the lower levels, before falling sharply (comparing observations of the same color in different exposure levels in Fig. 3b) at higher exposure levels. This "fall" happened at a lower exposure level for the isopods that ate 0 µg/L diet compared to the ones that ate 5 µg/L or the 500 µg/L diets. In the control (0 µg/L) Exposure level, 5 and 500 µg/L diet treatments reduced pigmentation change for a

given size, indicating that diet-mediated effects resulted in slightly lighter individuals.

4. Discussion

The presence of harmful chemicals in freshwater ecosystems is a growing environmental problem (Reid et al., 2019), with many of these chemicals being toxic to aquatic invertebrates, and adversely affecting freshwater ecosystems. However, while direct toxic effects are routinely assessed, indirect effects arising via altered resource base or species interactions are little studied. Here we tested both pathways of a less-investigated chemical cohort: fungicides (Zubrod et al., 2019). The primary findings indicate that while the different fungicides (Fluazinam, Tebuconazole, Urea) had partially linear to non-linear concentration-dependent effects – the direct vs indirect effects of Tebuconazole were trait dependent: growth was affected primarily via diet, feeding was affected additively via both direct and diet exposure, whereas effects of direct and indirect exposure on pigmentation were non-additive.

4.1. Range-finding: fungicides affect both growth and feeding rates of *A. aquaticus*

Since the mode of action and toxicity of fungicides can vary and have different impacts on organisms and ecosystems (Table A.1), we tested the sensitivity of *A. aquaticus* to three commonly used fungicides (Fluazinam, Tebuconazole, Urea). We found that Fluazinam had non-linear concentration dependent effects on *A. aquaticus* growth, and Tebuconazole and Urea had linear effects on growth and feeding, whereby Tebuconazole effects were strongest.

The observed nonlinear growth trend of *A. aquaticus* exposed to different concentrations of Fluazinam can be explained by the varying physiological responses at different concentrations. At low concentrations, a hormesis effect (Mattson, 2008) might occur, where slight stress from low exposure to a harmful substance results in seemingly positive effects such as enhanced growth but intermediate-high levels result in negative effects (Calabrese, 1999, Yearsley et al., 2004). At intermediate concentrations, the detoxification systems of *A. aquaticus* may become overloaded, leading to reduced growth rates (De Coninck et al., 2014). The energy cost of producing detoxification enzymes and repairing cellular damage can also divert resources from growth and reproduction, resulting in reduced growth rates. At higher concentrations, complex stress responses may occur, such as upregulation of additional detoxification pathways to cope with the increased toxic load (De Coninck et al., 2014), enabling the isopods to recover growth rates. It is possible that the responses of isopods in our study to Fluazinam reflect upregulation of detoxification and stress response genes, such as seen in Colorado potato beetle larvae (Saifullah et al., 2022). Alternatively, Fluazinam could act as an endocrine disruptor, interfering with hormonal regulation and affect important molecules crucial for molting and reproduction (Cui et al., 2017, Stenersen, 2004). Given such complexity of interactions between fungicides and physiological processes of organisms, further studies are needed to gain mechanistic understanding underlying the non-linear responses to Fluazinam.

The reason why we consider faster growth of individual isopods to have positive effects on the ecosystem, is because a larger isopod might feed more than a smaller one and thereby accelerate nutrient cycling of the aquatic detritus. However, it is important to note that these apparently positive effects on growth in response to Fluazinam did not translate into an increase in feeding rates in this experiment (Fig. 1). Hence, Fluazinam may increase isopod biomass (at least in the short-term) in the ecosystem, but without the associated functionality – the leaf decomposition rates would not be affected. Moreover, accelerated growth rates are often associated with trade-offs in other life-history traits, such as a shorter lifespan (Lee et al., 2013) or survival (Taddei et al., 2021), but such effects would not have been apparent in our 3-week study period under standardized laboratory conditions.

Fungicides have been shown to affect adult size through complex pathways (Margus et al., 2023), and the concentration-dependent decreases in growth rates caused by Tebuconazole were possibly because of fungicide toxicity and/or decreased nutritional quality of the diets. The increases in feeding rates may in part also reflect larger energetic needs (Feckler et al., 2016) and/or lower dietary quality (Cruz-Rivera and Hay, 2000). Here an increase in fungicide concentration may have eliminated a larger proportion of fungi from the leaves that the isopods feed on, and this affects the nutritional quality of their diets both due to lack of fungi as a food directly and by altering the palatability of the hard-to-digest leaf litter (Gardeström et al., 2016). This, in turn, would lead to the isopods receiving less energy from consuming leaves treated with fungicide, compared to untreated leaves (Feckler et al., 2016).

Urea had negative linear effects on *A. aquaticus* growth, indicating that high concentrations of urea do have negative effects even though Urea is considered environmentally safe. Larger isopods feeding more in Urea treatments (Fig.A.2) could be attributed to Urea being a fertilizer/nitrogen source, hence promoting microbial growth and enhancing food quality through that pathway. Even though the fungicidal effects would reduce diet quality, Urea may also have such positive effects (Huang et al., 2017). Hence, the effects of different fungicides can be either (non)linear and positive or negative, and the mechanisms for such patterns warrant further study.

4.2. Indirect (diet-mediated) effects of Tebuconazole on growth rates

We found that *A. aquaticus* growth was affected negatively by Diet, but not direct exposure, and in a size dependent manner – whereby larger individuals seemed to suffer more from dietary fungicide exposure (Fig. 2). This suggests that parts of the effects of fungicides in general, and Tebuconazole in particular, can arise via diet-mediated pathways. *A. aquaticus* growth rates are known to vary with diet quality (Lürig and Matthews, 2021), whereby changes in diet quality (such as via fungicides) can have strong effects on individual performance. In addition to changes in the fungal community and palatability of leaf litter, fungicide affected food would have larger amount of toxic compounds, and the resources now acquired go into detoxification and not growth.

Such direct and indirect effects of fungicides could have implications for overall ecosystem processes, since it is the larger isopods that contribute more to nutrient cycling by consuming most of the leaf litter (Vilisics et al., 2012), and since their body size is smaller than it should be, they would consume less detritus to maintain that body size. Also, smaller isopods are more vulnerable to predation (Sparkes, 1996), and this could also lead to indirect mortality since juveniles may mature at a smaller size and get eaten before they can reach higher rates of leaf consumption. While our individual level study doesn't directly assess population or ecosystem level consequences (Galic et al., 2018), population projection models using survival, growth, and reproduction data, along with larger-scale experiments, could bridge the gap to forecast how chronic fungicide exposure might impact long-term population dynamics. For instance, the reductions in growth and feeding rates observed here could translate into slower population growth or increased vulnerability to environmental stressors over time. Incorporating such models in future research would offer a more comprehensive understanding of the potential long-term impacts of fungicides on keystone species like *A. aquaticus*.

4.3. Direct and diet-mediated Tebuconazole exposure can reduce feeding rates

Our results indicate that feeding rates decreased additively with increasing direct exposure and diet concentrations. The effect of direct exposure on feeding rates could be due to toxicity (Zubrod et al., 2011) combined with altered food quality, or due to changes in microbiomes which may reduce efficient assimilation (Kish et al., 2013) - especially in

isopods that are known to harbour microbiomes with likely beneficial function (Bouchon et al., 2016), including *A. aquaticus* (Bredon et al., 2020, Lafuente et al., 2023).

It is alarming that even the lowest level of Tebuconazole exposure in our experiment (5 μg/L), which is lower than the environmentally relevant levels (Wang et al., 2018, Baudy-Groh et al., 2021), significantly reduced feeding rates and, hence, may also affect ecosystem functioning on a larger scale. The possible bioaccumulation and concentration of toxins may also be concerning, because even though *A. aquaticus* is generally tolerant to chemical pollution (O'Callaghan et al., 2019), it has several trophic links to other species which may be more sensitive (Lafuente et al., 2021).

A. aquaticus has been shown to prefer naturally conditioned leaves with their own bacterial and fungal communities (Bloor, 2011), and fungicide-treated leaves may lack the vital fungal component (Artigas et al., 2012) resulting in lower feeding rates in fungicide-treated leaf litter (e.g. Rocha Dimitrov et al., 2014, Feckler et al., 2016). At the same time, the elevated feeding rates seen in the 5 μg/L diet (relative to 0 μg/L diet control) in some of the direct exposure concentrations in our study, could be explained as a lower energy utilization efficiency (Sokolova et al., 2012, Van Ginneken et al., 2019). It is also possible that at these low (~5 μg/L) levels, the fungicide might suppress parasitic fungi affecting the isopods (Machado et al., 2022) and cause an apparent positive effect (Fig. 3a), while after a certain threshold, the negative effects may outweigh such a positive effect and cause reduced feeding.

4.4. Interactive effects of Tebuconazole treated diets and direct exposure on pigmentation

Cryptic pigmentation is an important trait in prey species, including *A. aquaticus* (Hargeby et al., 2005), but less studied in the context of ecotoxicological responses. In contrast to diet-mediated effects on growth, and additive effects of direct and indirect pathways on feeding rates, we found that direct and diet-mediated pathways of Tebuconazole had interactive effects on pigmentation change: At higher Exposure levels (50 and 500 μg/L), isopods became relatively lighter, but the threshold where the abrupt fall in pigmentation happens, and the severity seemed to depend on the diet the isopods consume. The isopods that ate the 0 μg/L treated Diet became lighter in 50 μg/L Exposure, but the ones that ate 5 μg/L and 500 μg/L treated Diets, became lighter in 500 μg/L Exposure level (Fig. 3b). While the mechanism behind such interactive effects is currently unknown, fungicides may disrupt physiological processes or resource assimilation, so that the isopods are unable to produce the costly dark pigment (Lürig et al., 2019). While our study did not specifically investigate the biochemical pathways involved, existing literature on the biochemistry and biosynthesis of insect pigments suggests that disruptions in the tryptophan metabolic pathway can affect pigmentation processes (Shamim et al., 2014). Tryptophan is an essential amino acid which can only be obtained from the diet and is the precursor molecule in the developmental pathway of *A. aquaticus*' pigmentation. As Tebuconazole may inhibit fungal enzymes and alter fungal metabolism, it may impact the availability and metabolism of tryptophan in the food of *A. aquaticus*. Alternatively, but not mutually exclusive, Tebuconazole may also affect the isopods directly by inhibiting key enzymes in the tryptophan metabolic pathway, which is crucial for the synthesis of pigments such as ommochromes.

In general, there is a positive correlation between growth and pigmentation in *A. aquaticus* (Hargeby et al., 2005; Lürig and Matthews, 2021), both due to genetic effects (Eroukhmanoff et al., 2009) and via phenotypic plasticity (Lürig et al., 2019). Because pigmentation is important for *A. aquaticus* to avoid visual-based predation (Rask and Hiisivuori, 1985), any effects of environmental contaminants – either directly or via diet – on their ability to adjust pigmentation can have detrimental effects in nature. *A. aquaticus* vary in pigmentation depending on substrate (Eroukhmanoff et al., 2009, Lürig et al., 2019), with selection favouring matching the environment, such as maintaining dark pigmentation while on detritus on the lake floor and lighter in reed habitats in presence of visual predators such as fish (Lürig et al., 2019). Moreover, darker individuals tend to survive UV exposure better than their lighter counterparts (Johansson, 2005), which might be particularly important in shallow areas of Nordic clear water lakes, such as lake Konnevesi, the source of our study population. Hence, in environments with a dark substrate or elevated UV levels, fungicides causing isopods to exhibit lighter pigmentation might cause a mismatch in coloration and expose them to visually foraging predators – or less protection from harmful UV radiation, but further tests would be needed to assess this.

5. Conclusion

This study investigated the sensitivity of the freshwater detritivore *Asellus aquaticus* to three common fungicides, and the potential for direct and indirect effects of fungicides on individual growth, food consumption, and pigmentation. Our results indicate that fungicides negatively impact these key traits through direct toxicity, indirect (diet-mediated) pathways, and interactive effects. Such individual level sublethal effects can also be detrimental, potentially making *A. aquaticus* more vulnerable to predation and UV radiation due to reduced pigmentation (Johansson, 2005). While not easy to directly infer, even sublethal responses of a keystone species like *A. aquaticus* could further impact important ecosystem processes, such as nutrient cycling (due to reduced growth and feeding rates), and cascade through the entire food web (Galic et al., 2018). Longer term experiments in more ecologically realistic settings would shed light on how individual level responses to fungicides transfer to other trophic levels (Zubrod et al., 2019). In conclusion, in addition to well-known direct effects of chemical pollution, it is crucial to consider multiple impact pathways – such as those mediated through diet - to fully understand the ecological consequences of anthropogenic stressors.

CRediT authorship contribution statement

Akshay Mohan: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Blake Matthews:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Katja Räsänen:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. : Range-finding

Table A.1
More information on the fungicides used in range-finding assay

Fungicide	Modes of Action	Environmental Relevance
Fluazinam	Causes disruption of energy production in fungi at multiple sites	Used in agriculture to combat molds and blights that can affect crops (e.g. potatoes) Has the highest ecotoxicological impact among fungicides in Finland (Räsänen et al., 2014). Induces oxidative stress in non-target aquatic organisms by disrupting normal cellular respiration processes, leading to increased reactive oxygen species (ROS) that can damage cellular structures and impair function. Shown to have acute toxicity to aquatic organisms and gets bioaccumulated in aquatic invertebrates such as Daphnia (Wu et al., 202)
Tebuconazole	Acts as a demethylation inhibitor, suppresses spore germination and growth and interferes with ergosterol production which is essential for building fungal cell walls	Commonly used in agriculture on crops (e.g. cereals and fruits) to control powdery mildew, rusts, and leaf spots (European Food Safety Authority, 2014) Acts as an endocrine disruptor in non-target aquatic species, affecting hormone regulation which can lead to altered development, behavior, and reproduction in fish and amphibians (e.g. zebrafish have shown clear signs of poisoning, loss of movement coordination, dullness, and pale color (Sancho et al., 2009))
Urea	Releases ammonia and increases pH to a level that is toxic to fungi	The most used fungicide in Finland against *Annosum* root rot disease in forestry (Finnish Safety and Chemicals Agency (Tukes) website) Considered ecotoxicologically low-risk (Alvarez-Alfageme et al., 2023) but can result in toxic ammonia spikes. Excessive urea can also lead to eutrophication, reducing oxygen availability and causing stress or mortality in aquatic organisms

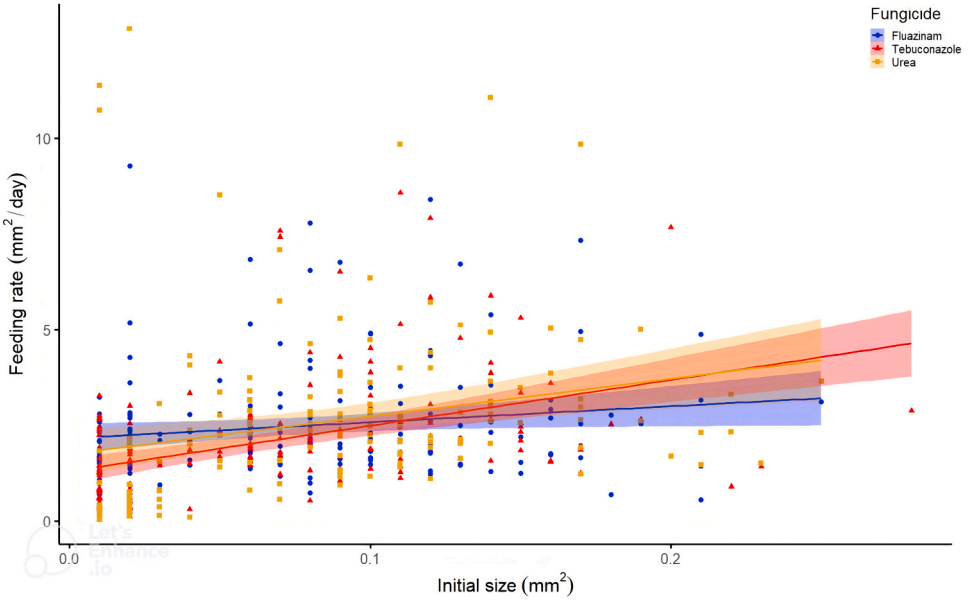

Figure A.1. This scatter plot with linear regression lines shows the relationship between initial body size (mm^2) and feeding rate (mm^2/day) of juvenile *Asellus aquaticus* across different fungicides Raw data alongside the respective 95 % confidence intervals is plotted here, with each point presenting the values for individual *A. aquaticus*. The positive slopes indicate the isopods with larger initial size showed a faster feeding rate.

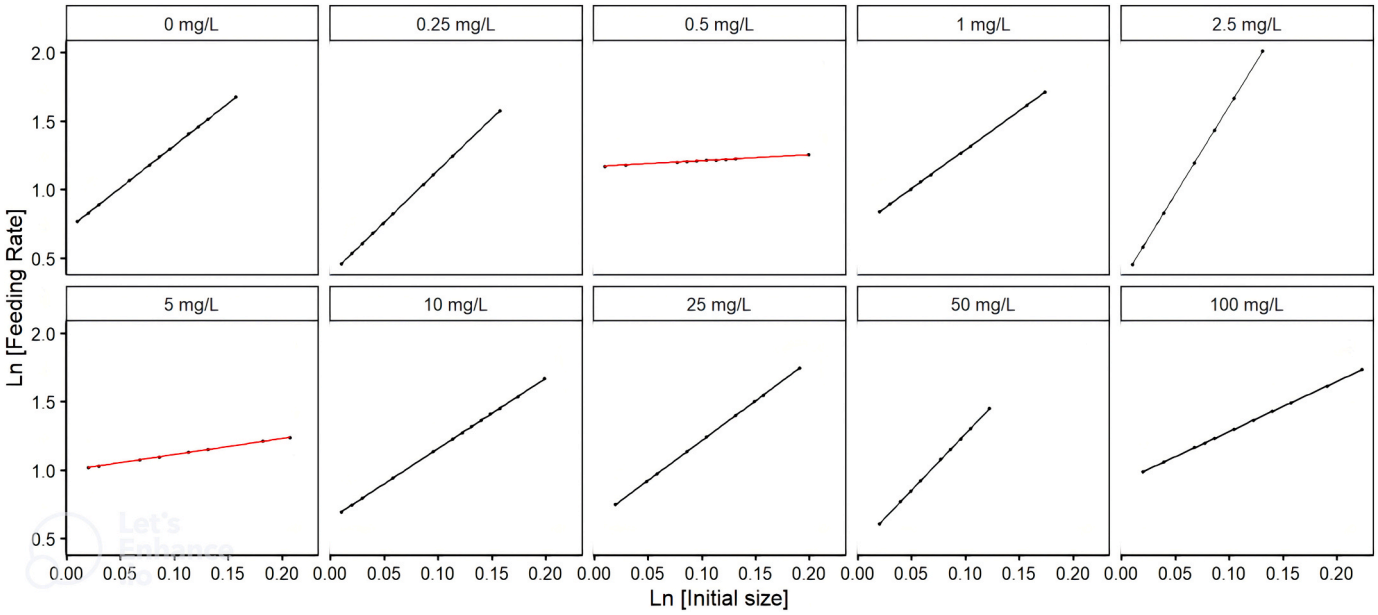

Figure A.2. Relationship between initial size and feeding rate (both log-transformed) at various urea concentrations. The regression lines for 0.5 mg/L and 5 mg/L urea concentrations are highlighted in red to indicate the slopes being different from other concentrations. The positive slopes indicate that the respective Urea concentrations increased feeding rates of larger isopods.

Protocol A.1. Leaf Disc Preparation and Microbial Colonization

Initially, dry *Alnus glutinosa* leaves were soaked in animal-safe ground water for three days. Following this soaking period, leaf discs were made using a 10 mm leaf punch tool, carefully creating uniform discs which were then spread out on a metal tray to avoid overlap. These discs underwent a sterilization process under UV light for 30 minutes, exposed for 15 minutes on one side, then flipped and exposed for another 15 minutes on the opposite side to ensure complete sterilization. Immediately afterwards, the sterilized leaf discs were inoculated in lakewater from lake Konnevesi which would have the essential microbes, for seven days. This immersion allowed for the natural colonization of the leaf surfaces by lake water microbes, setting the stage for our investigations into microbial community dynamics on leaf surfaces. This preparation protocol was crucial for our study, ensuring that the leaf discs were both sterilized and then exposed to a controlled microbial colonization environment.

Appendix B. : Direct and Indirect effects of Tebuconazole

Table B.1
Slopes (Growth rate vs Initial size) from Linear Mixed Effects Model in which growth rate was analyzed with diet, exposure and their interactions as fixed effects, initial body size as covariate, and plate number as random effect. From the model, 5 µg/L and 500 µg/L diet levels showed a smaller slope compared to the 0 µg/L level, which shows a positive relationship between Growth rate and Initial size.

Diet	*Slope*	*SE*	*df*	*P*
0 µg/L	0.192	0.050	191	**<0.001**
5 µg/L	0.050	0.082	204	0.542
500 µg/L	0.025	0.055	201	0.651

Table B.2
Average feeding rates (predicted means) were calculated from Linear Mixed Effects Model in which feeding rate was analyzed with diet, exposure and their interactions as fixed effects, initial body size as covariate, and plate number as random effect. To determine the percentage changes in feeding rates in the different fungicide treatments, these values were then back transformed using the formula: *Feeding rate = exp(predicted mean) − constant*

*Treatment (Exposure * Diet)*	Ln [Feeding rate] ± SE (Predicted means)	*Df*	*Feeding rate (mm^2/day)*
0 µg/L * 0 µg/L	−0.034 ± 0.120	49.5	0.936
0 µg/L * 5 µg/L	−0.004 ± 0.131	60.8	0.966
0 µg/L * 500 µg/L	−0.272 ± 0.137	64.0	0.732
5 µg/L * 0 µg/L	−0.112 ± 0.122	51.4	0.863
5 µg/L * 5 µg/L	−0.176 ± 0.144	66.6	0.808

(*continued on next page*)

Table B.2 (*continued*)

*Treatment (Exposure * Diet)*	Ln [Feeding rate] ± SE (Predicted means)	*Df*	*Feeding rate (mm^2/day)*
5 µg/L * 500 µg/L	−0.387± 0.124	53.1	0.649
50 µg/L * 0 µg/L	−0.385 ± 0.122	50.9	0.650
50 µg/L * 5 µg/L	−0.219 ± 0.126	54.8	0.772
50 µg/L * 500 µg/L	−0.419 ± 0.124	52.7	0.627
500 µg/L * 0 µg/L	−0.462 ± 0.120	49.3	0.599
500 µg/L * 5 µg/L	−0.413 ± 0.122	50.9	0.631
500 µg/L * 500 µg/L	−0.701 ± 0.122	51.2	0.465

References

Abramoff, M., Magalhães, P., Ram, S.J., 2004. Image Processing with ImageJ. Biophoton. Int. 11, 36–42.

Alvarez-Alfageme, F., Arena, M., Auteri, D., Leite, S., Binaglia, M., Castoldi, A., Chiusolo, A., Cioca, A.-A., Colagiorgi, A., Colas, M., Crivellente, F., Lentdecker, C., Magistris, I., Egsmose, M., Fait, G., Ferilli, F., Gouliarmou, V., Halling, K., Nogareda, L., Villamar Bouza, L., 2023. Peer review of the pesticide risk assessment of the active substance urea. EFSA J. 21. https://doi.org/10.2903/j.efsa.2023.8112.

Artigas, J., Majerholc, J., Foulquier, A., Margoum, C., Volat, B., Neyra, M., Pesce, S., 2012. Effects of the fungicide tebuconazole on microbial capacities for litter breakdown in streams. Aquat. Toxicol. (Amst., Neth.) 122-123, 197–205. https://doi.org/10.1016/j.aquatox.2012.06.011.

Baudy-Groh, P., Zubrod, J., Konschak, M., Röder, N., Nguyen, T., Schreiner, V., Baschien, C., Schulz, R., Bundschuh, M., 2021. Environmentally relevant fungicide levels modify fungal community composition and interactions but not functioning. Environ. Pollut. 285, 117234. https://doi.org/10.1016/j.envpol.2021.117234.

Berenzen, N., Lentzen-Godding, A., Probst, M., Schulz, H., Schulz, R., Liess, M., 2005. A comparison of predicted and measured levels of runoff-related pesticide concentrations in small lowland streams on a landscape level. Chemosphere 58, 683–691. https://doi.org/10.1016/j.chemosphere.2004.05.009.

Bloor, M., 2011. Dietary preference of *Gammarus pulex* and *Asellus aquaticus* during a laboratory breeding programme for ecotoxicological studies. Int. J. Zool. 2011. https://doi.org/10.1155/2011/294394.

Bouchon, D., Zimmer, M., Dittmer, J., 2016. The terrestrial isopod microbiome: an all-in-one toolbox for animal–microbe interactions of ecological relevance. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.01472.

Bredon, M., Herran, B., Bertaux, J., Grève, P., Moumen, B., Bouchon, D., 2020. Isopod holobionts as promising models for lignocellulose degradation. Biotechnol. Biofuels 13. https://doi.org/10.1186/s13068-020-01683-2.

Bundschuh, M., Zubrod, J., Kosol, S., Maltby, L., Stang, C., Duester, L., Schulz, R., 2011. Fungal composition on leaves explains pollutant-mediated indirect effects on amphipod feeding. Aquat. Toxicol. 104, 32–37. https://doi.org/10.1016/j.aquatox.2011.03.010.

Calabrese, E., 1999. Evidence that hormesis represents an "overcompensation" response to a disruption in homeostasis. Ecotoxicol. Environ. Saf. 42, 135–137. https://doi.org/10.1006/eesa.1998.1729.

Caseiro, I., Santos, S., Sousa, J.P., Nogueira, A., Soares, A., 2000. Optimization of culture conditions of *Porcellio dilatatus* (Crustacea: Isopoda) for laboratory test development. Ecotoxicol. Environ. Saf. 47, 285–291. https://doi.org/10.1006/eesa.2000.1982.

Cruz-Rivera, E., Hay, M., 2000. Can quantity replace quality? Food choice, compensatory feeding, and fitness of marine mesograzers. Ecology 81. https://doi.org/10.1890/0012-9658(2000)081[0201:CQRQFC]2.0.CO;2.

Cui, F., Chai, T., Liu, X., Wang, C., 2017. Toxicity of three strobilurins (kresoxim-methyl, pyraclostrobin, and trifloxystrobin) on *Daphnia magna*. Environ. Toxicol. Chem. 36, 182–189. https://doi.org/10.1002/etc.3520.

De Coninck, D., Asselman, J., Glaholt, S., Janssen, C., Colbourne, J.K., Shaw, J., De Schamphelaere, K., 2014. Genome-wide transcription profiles reveal genotype-dependent responses of biological pathways and gene-families in *Daphnia* exposed to single and mixed stressors. Environ. Sci. Technol. 48 (6), 3513–3522. https://doi.org/10.1021/es4053363.

Elsaesser, D., Schulz, R., 2008. Mitigation of fungicide pollution in vegetated agricultural surface waters: GIS modelling and monitoring in the field. Proc. SETAC Eur. 18th Annu. Meet. 406–407.

Eroukhmanoff, F., Hargeby, A., Svensson, E., 2009. Rapid adaptive divergence between ecotypes of an aquatic isopod inferred from FST-QST analyses. Mol Ecol 18: 4912-4923. Mol. Ecol. 18, 4912–4923. https://doi.org/10.1111/j.1365-294X.2009.04408.x.

European Chemicals Agency. (2023). Tebuconazole: Data on environmental degradation. Retrieved from [ECHA website] (⟨https://echa.europa.eu/documents/10162/b02f4de6-574e-6ba3-7f80-af8ea03df463⟩).

European Food Safety Authority. (2014). Conclusion on the peer review of the pesticide risk assessment of the active substance tebuconazole. EFSA Journal, 12. https://doi.org/10.2903/j.efsa.2014.3485.

Feckler, A., Goedkoop, W., Zubrod, J., Schulz, R., Bundschuh, M., 2016. Exposure pathway-dependent effects of the fungicide epoxiconazole on a decomposer-detritivore system. Sci. Total Environ. 571, 992–1000. https://doi.org/10.1016/j.scitotenv.2016.07.088.

Finlay, K., Patoine, A., Donald, D., Bogard, M., Leavitt, P., 2010. Experimental evidence that pollution with urea can degrade water quality in phosphorus-rich lakes of the Northern Great Plains. Limnol. Oceanogr. 55, 1213–1230. https://doi.org/10.4319/lo.2010.55.3.1213.

Finnish Safety and Chemicals Agency (Tukes). (n.d.). Safe use of plant protection products - Harmonised risk indicators. Retrieved from ⟨https://tukes.fi/en/safe-use-of-plant-protection-products/harmonised-risk-indicators⟩.

Galic, N., Sullivan, L.L., Grimm, V., Forbes, V.E., 2018. When things don't add up: quantifying impacts of multiple stressors from individual metabolism to ecosystem processing. Ecol. Lett. 21 (4), 568–577. https://doi.org/10.1111/ele.12923.

Gardeström, J., Ermold, M., Goedkoop, W., Mckie, B., 2016. Disturbance history influences stressor impacts: effects of a fungicide and nutrients on microbial diversity and litter decomposition. Freshw. Biol. 61. https://doi.org/10.1111/fwb.12698.

Graça, M., Maltby, L., Calow, P., 1993. Importance of fungi in the diet of *Gammarus pulex* and *Asellus aquaticus* I: feeding strategies. Oecologia 93, 139–144. https://doi.org/10.1007/BF00321203.

Graça, M., Maltby, L., Calow, P., 1994. Comparative ecology of *Gammarus pulex* (L.) and *Asellus aquaticus* (L.) II: Fungal preferences. Hydrobiologia 281, 163–170. https://doi.org/10.1007/BF00028679.

Groh, K., Berg, C., Schirmer, K., Tlili, A., 2022. Anthropogenic chemicals as underestimated drivers of biodiversity loss: Scientific and societal implications. Environ. Sci. Technol. 56 (2), 707–710. https://doi.org/10.1021/acs.est.1c08399.

Hakala, K., Yli-Halla, M., Tuomainen, P., Hartikainen, H., 2020. Persistence of fluazinam in soil under boreal conditions. J. Environ. Sci. Health, Part B 55, 1–11. https://doi.org/10.1080/03601234.2020.1729631.

Hargeby, A., Stoltz, J., Johansson, J., 2005. Locally differentiated cryptic pigmentation in the freshwater isopod *Asellus aquaticus*. J. Evolut. Biol. 18, 713–721. https://doi.org/10.1111/j.1420-9101.2004.00837.x.

Hopkins, A., Hoverman, J., 2023. Acute aquatic toxicity of two commonly used fungicides to midwestern amphibian larvae. Ecotoxicology 32, 1–8. https://doi.org/10.1007/s10646-023-02629-8.

Huang, W., Gao, Y., Xiao, Y., Maberly, S.C., Gontero, B., 2017. Effects of urea on growth and photosynthetic metabolism of two submersed aquatic plants. Aquat. Bot. 140, 69–77. https://doi.org/10.1016/j.aquabot.2016.04.003.

Johansson, J. (2005). Local adaptive pigmentation in *Asellus aquaticus* -effects of UV-radiation and predation regime. [Doctoral Thesis (compilation), Department of Biology]. Limnology, Department of Ecology, Lund University.

Kish, L., Hotte, N., Kaplan, G.G., Vincent, R., Tso, R., Gänzle, M., Rioux, K.P., Thiesen, A., Barkema, H.W., Wine, E., Madsen, K.L., 2013. Environmental particulate matter induces murine intestinal inflammatory responses and alters the gut microbiome. PLoS One 8 (4), e62220. https://doi.org/10.1371/journal.pone.0062220.

Lafuente, E., Lürig, M., Rövekamp, M., Matthews, B., Buser, C., Vorburger, C., Räsänen, K., 2021. Building on 150 years of knowledge: the freshwater isopod *Asellus aquaticus* as an integrative eco-evolutionary model system. Front. Ecol. Evol. 9. https://doi.org/10.3389/fevo.2021.748212.

Lafuente, E., Carles, L., Walser, J., Giulio, M., Wullschleger, S., Stamm, C., Räsänen, K., 2023. Effects of anthropogenic stress on hosts and their microbiomes: treated wastewater alters performance and gut microbiome of a key detritivore (*Asellus aquaticus*) (n/a-n/a). Evolut. Appl. 16. https://doi.org/10.1111/eva.13540.

Lee, W.-S., Monaghan, P., Metcalfe, N., 2013. Experimental demonstration of the growth rate - lifespan trade-off. Proc. R. Soc. B: Biol. Sci. 280. https://doi.org/10.1098/rspb.2012.2370.

Lürig, M., Best, R., Svitok, M., Jokela, J., Matthews, B., 2019. The role of plasticity in the evolution of cryptic pigmentation in a freshwater isopod. J. Anim. Ecol. 88. https://doi.org/10.1111/1365-2656.12950.

Lürig, M. (2021). phenopype: a phenotyping pipeline for Python. Methods in Ecology and Evolution. https://doi.org/10.1101/2021.03.17.435781.

Lürig, M., Matthews, B., 2021. Dietary-based developmental plasticity affects juvenile survival in an aquatic detritivore. Proc. R. Soc. B: Biol. Sci. 288, 20203136. https://doi.org/10.1098/rspb.2020.3136.

Machado, C., Cuco, A., Cassio, F., Wolinska, J., Castro, B., 2022. Antiparasitic potential of agrochemical fungicides on a non-target aquatic model (*Daphnia* × *Metschnikowia* host-parasite system). Sci. Total Environ. 833, 155296. https://doi.org/10.1016/j.scitotenv.2022.155296.

Maltby, L., Brock, T., Van den Brink, P., 2009. Fungicide risk assessment for aquatic ecosystems: Importance of interspecific variation, toxic mode of action, and exposure regime. Environ. Sci. Technol. 43, 7556–7563. https://doi.org/10.1021/es901461c.

Marcus, J., Willoughby, L., 1978. Fungi as food for the aquatic invertebrate *Asellus aquaticus*. Trans. Br. Mycol. Soc. 70, 143–146. https://doi.org/10.1016/S0007-1536(78)80186-7.

Margus, A., Saifullah, S., Kankare, M., Lindström, L., 2023. Fungicides modify pest insect fitness depending on their genotype and population. Sci. Rep. 13. https://doi.org/10.1038/s41598-023-44838-5.

Mattson, M., 2008. Hormesis Defined. Ageing Res. Rev. 7, 1–7. https://doi.org/10.1016/j.arr.2007.08.007.

Noori, R., Woolway, R., Jun, C., Bateni, S., Maghrebi, M., Pulkkanen, M., 2022. Multi-decadal change in summer mean water temperature in Lake Konnevesi. Finland 1984–2021. https://doi.org/10.21203/rs.3.rs-1982578/v1.

O'Callaghan, I., Harrison, S.S.C., Fitzpatrick, D., Sullivan, T., 2019. The freshwater isopod *Asellus aquaticus* as a model biomonitor of environmental pollution: a review. Chemosphere 235. https://doi.org/10.1016/j.chemosphere.2019.06.217.

Räsänen, K., Mattila, T., Porvari, P., Kurppa, S., Tiilikkala, K., 2014. Estimating the development of ecotoxicological pressure on water systems from pesticides in Finland 2000–2011. J. Clean. Prod. 89, 65–77. https://doi.org/10.1016/j.jclepro.2014.11.008.

Rask, M., Hiisivuori, C., 1985. The predation on *Asellus aquaticus* (L.) by perch, *Perca fluviatilis* (L.), in a small forest lake. Hydrobiologia 121, 27–33. https://doi.org/10.1007/BF00035226.

R Core Team. (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Retrieved from ⟨https://www.R-project.org/⟩.

Reid, A.J., Carlson, A.K., Creed, I.F., Eliason, E.J., Gell, P.A., Johnson, P.T.J., Kidd, K.A., MacCormack, T.J., Olden, J.D., Ormerod, S.J., Smol, J.P., Taylor, W.W., Tockner, K., Vermaire, J.C., Dudgeon, D., Cooke, S.J., 2019. Emerging threats and persistent conservation challenges for freshwater biodiversity. Biol. Rev. 94, 849–873. https://doi.org/10.1111/brv.12480.

Rocha Dimitrov, M., Kosol, S., Smidt, H., Buijse-Bogdan, L., Van den Brink, P., Wijngaarden, R., Brock, T., Maltby, L., 2014. Assessing effects of the fungicide tebuconazole to heterotrophic microbes in aquatic microcosms. Sci. Total Environ. 490C, 1002–1011. https://doi.org/10.1016/j.scitotenv.2014.05.073.

Rossi, L., Fano, E., 1979. Role of fungi in the trophic niche of the congeneric detritivorous *A. aquaticus and A. coxalis* (Isopoda). Oikos 32, 380–385. https://doi.org/10.2307/3544749.

Saifullah, S., Margus, A., Kankare, M., Lindström, L., 2022. Repeated exposure of fluazinam fungicides affects gene expression profiles yet carries no costs on a non-target pest. Insect Sci. 29. https://doi.org/10.1111/1744-7917.13013.

Sancho, E., M.J., Fernández, C., Andreu-Moliner, E., Ferrando, M., 2009. Short-term exposure to sublethal tebuconazole induces physiological impairment in male zebrafish (*Danio rerio*). Ecotoxicol. Environ. Saf. 73, 370–376. https://doi.org/10.1016/j.ecoenv.2009.09.020.

Shamim, G., Ranjan, S.K., Pandey, D.M., Ramani, R., 2014. Biochemistry and biosynthesis of insect pigments. EJE 111 (2), 149–164. https://doi.org/10.14411/eje.2014.021.

Shikuku, V., Kowenje, C., Ongeri, D., Zanella, R., Prestes, O., 2014. Removal of Tebuconazole from wastewater by Zeolite X: kinetics and thermodynamics studies. Int. J. Eng. Res. Technol. (IJERT) 3, 1584–1590. https://doi.org/10.17577/IJERTV3IS080860.

Sokolova, I., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A., 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar. Environ. Res. 79, 1–15. https://doi.org/10.1016/j.marenvres.2012.04.003.

Sparkes, T., 1996. Effects of predation risk on population variation in adult size in a stream-dwelling isopod. Oecologia 106, 85–92. https://doi.org/10.1007/BF00334410.

Stamm, C., Räsänen, K., Burdon, F.J., Altermatt, F., Jokela, J., Joss, A., M. Ackermann Eggen, R.I.L., 2016. Unravelling the impacts of micropollutants in aquatic ecosystems: interdisciplinary studies at the interface of large-scale ecology. Adv. Ecol. Res. 55, 183–223. https://doi.org/10.1016/bs.aecr.2016.07.002.

Stenersen, J., 2004. Chemical pesticides: Mode of action and toxicology. CRC Press. https://doi.org/10.1201/9780203646830.

Taddei, A., Räsänen, K., Burdon, F., 2021. Size-dependent sensitivity of stream amphipods indicates population-level responses to chemical pollution. Freshw. Biol. 00, 1–20. https://doi.org/10.1111/fwb.13677.

Tennakoon, D., Gentekaki, E., Jeewon, R., Kuo, C.-H., Promputtha, I., Hyde, K., 2021. Life in leaf litter: Fungal community succession during decomposition. Mycosphere 12, 406–429. https://doi.org/10.5943/mycosphere/12/1/5.

Tofan, L., Niţă, V., Nenciu, M., Valentina, C., Lazar, L., Nicoleta, D., Vasile, D., Popoviciu, D., Brotea, A., Bănăduc, A., Avramescu, S., Aonofriesei, F. (2023). Multiple Assays on Non-Target Organisms to Determine the Risk of Acute Environmental Toxicity in Tebuconazole-Based Fungicides Widely Used in the Black Sea Coastal Area. Toxics. 11. 597. https://doi.org/10.3390/toxics11070597.

U.S. Environmental Protection Agency. (2001). Fluazinam: Pesticide Fact Sheet. Retrieved from [EPA website] (⟨https://www3.epa.gov/pesticides/chem_search/reg_actions/registration/fs_PC-129098_10-Aug-01.pdf⟩).

Urbańczyk, E., Sowa, M., Simka, W., 2016. Urea removal from aqueous solutions—a review. J. Appl. Electrochem 46, 1011–1029. https://doi.org/10.1007/s10800-016-0993-6.

Van Ginneken, M., Blust, R., Bervoets, L., 2018. Combined effects of metal mixtures and predator stress on the freshwater isopod *Asellus aquaticus*. Aquat. Toxicol. 200, 148–157.

Van Ginneken, M., Blust, R., Bervoets, L., 2019. The impact of temperature on metal mixture stress: sublethal effects on the freshwater isopod *Asellus aquaticus*. Environ. Res. 169. https://doi.org/10.1016/j.envres.2018.10.025.

Vilisics, F., Szekeres, S., Hornung, E., 2012. Size-dependent differences in litter consumption of isopods: preliminary results. ZooKeys 247–259. https://doi.org/10.3897/zookeys.176.2470.

Wang, X., Zheng, S., Huang, T., Su, L., Zhao, Y., Souders, I.I., C., Martyniuk, C., 2018. Fluazinam impairs oxidative phosphorylation and induces hyper/hypo-activity in a concentration specific manner in zebrafish larvae. Chemosphere 210. https://doi.org/10.1016/j.chemosphere.2018.07.056.

Wang, X., Li, X., Wang, Y., Qin, Y., Yan, B., Martyniuk, C., 2021. A comprehensive review of strobilurin fungicide toxicity in aquatic species: emphasis on mode of action from the zebrafish model. Environ. Pollut. 275, 116671. https://doi.org/10.1016/j.envpol.2021.116671.

Wu, W., Kong, D., Zhang, W., Bu, Y., Li, J., Shan, Z., 2020. Acute toxicity of fluazinam to aquatic organisms and its bioaccumulation in *BrachyDanio rerio*. Environ. Sci. Pollut. Res. 27, 1–8. https://doi.org/10.1007/s11356-020-09779-w.

Yao, C., Li, C., Nie, D., Chen, Y., Guo, X., Cao, N., Li, X., Pang, S., 2022. Exposure to fenvalerate and tebuconazole exhibits combined acute toxicity in zebrafish and behavioral abnormalities in larvae. Front. Environ. Sci. 10, 975634. https://doi.org/10.3389/fenvs.2022.975634.

Yearsley, J.M., Kyriazakis, I., Gordon, I.J., 2004. Delayed costs of growth and compensatory growth rates. Funct. Ecol. 18, 563–570. https://doi.org/10.1111/j.0269-8463.2004.00879.x.

Zubrod, J., Bundschuh, M., Feckler, A., Englert, D., Schulz, R., 2011. Ecotoxicological impact of the fungicide tebuconazole on an aquatic decomposer-detritivore system. Environ. Toxicol. Chem. / SETAC 30, 2718–2724. https://doi.org/10.1002/etc.679.

Zubrod, J., Bundschuh, M., Arts, G., Brühl, C., Imfeld, G., Knäbel, A., Payraudeau, S., Rasmussen, J., Rohr, J., Scharmüller, A., Smalling, K., Stehle, S., Schulz, R., Schäfer, R., 2019. Fungicides: an overlooked pesticide class? Environ. Sci. Technol. 53. https://doi.org/10.1021/acs.est.8b04392.