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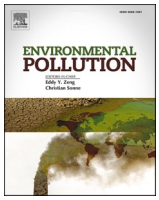

# Locomotor activity and physiological responses of parasite-infected *Gammarus fossarum* exposed to the herbicide metazachlor☆

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ABSTRACT

Herbicides are among the most commonly found contaminants in freshwater ecosystems. Standard tests are frequently employed to assess their ecotoxicological impacts, but sublethal endpoints in non-target species are often not considered. In addition, ecotoxicological investigations rarely take into account that many species from field populations are naturally infected with parasites. To overcome these gaps, our study aimed to investigate how environmentally relevant concentrations of the herbicide metazachlor affect the locomotor activity and selected physiological responses of *Gammarus fossarum* infected with the acanthocephalan *Polymorphus minutus* and microsporidians. Prior to the study of sublethal effects, acute immobility, and lethality (EC50 and LC50) tests were conducted. *Polymorphus minutus*, but not microsporidians, slightly enhanced chemical stress tolerance in infected *G. fossarum* in the acute immobility and lethality test. Infections with *P. minutus* significantly increased the host's locomotory activity in comparison to uninfected individuals when exposed to environmentally relevant concentrations of metazachlor, while metazachlor exposure alone had no apparent impact on locomotion. In contrast, the effects of metazachlor on physiological responses (glutathione S-transferase, glycogen, and phenoloxidase) of *G. fossarum* were significant, while parasite infection alone did not exhibit any significant impact on these biomarkers. The findings of our study indicate that the locomotor activity of *G. fossarum* in the conducted exposure tests was mostly influenced by *P. minutus* infections. Conversely, physiological responses were predominantly associated with exposure to metazachlor at environmentally relevant concentrations. We recommend future ecotoxicological studies involving non-target, field-collected species to consider the potential bias introduced by parasitic infections to ensure accurate evaluations of the effects of environmental contaminants.

## 1. Introduction

Herbicides are increasingly being detected in aquatic ecosystems, raising environmental concerns (Brêda-Alves et al., 2021; Vonk and Kraak, 2020). Although designed to control unwanted vegetation, herbicides often contaminate aquatic environments via runoff, leaching, or spray drift (Brühl and Zaller, 2021; Shefali et al., 2021) affecting a wide range of non-target organisms (Pinheiro et al., 2023; Vonk and Kraak, 2020). Among the high numbers of herbicides in use today, metazachlor (MTZ), a chloroacetamide herbicide, is frequently detected in surface waters at levels of 0.1 μg/L – 100 μg/L (Guo et al., 2021; Mohr et al., 2008). MTZ may negatively impact aquatic species by inhibiting long-chain fatty acid synthesis, thereby interfering with cell division and tissue differentiation during growth (Park et al., 2020; Salač et al., 2019). Its primary agricultural use includes controlling pre- and early post-emergence of annual grasses and broadleaf weeds (Karier et al.,

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2017; Velisek et al., 2020). Being a widely used herbicide, the ecotoxicological effects of MTZ have been studied using standard test species, including plants (Müller et al., 2010; Wijewardene et al., 2021) and aquatic organisms. For instance, in fish, the 96h LC50 of MTZ for rainbow trout *Oncorhynchus mykiss*, bluegill sunfish *Lepomis macrochirus*, and common carp *Cyprinus carpio* are 4.0 mg/L, 15.0 mg/L, and 15.0 mg/L, respectively, while the 72h EC50 for the green algae *Pseudokirchneriella subcapitata* is 0.031 mg/L, and the 96h EC50 for *Chlorella* spp. is 1.63 mg/L (FAO, 1999). However, standardized tests usually include only a limited number of test species (Taylor and Scroggins, 2013; US EPA, 1994). Consequently, the effects of MTZ on most freshwater invertebrates have not yet been adequately investigated (Velisek et al., 2020). Besides, standard ecotoxicological tests for risk assessments often use effective or lethal concentration (EC or LC) of contaminants, which differ from what organisms encounter in natural conditions (Alves and Cardoso, 2016; Singla et al., 2021); thereby, the chronic effects at environmentally relevant concentrations are underestimated (Posthuma et al., 2019). Sublethal endpoints can provide valuable insights into exposed organisms' overall health, indicating early warning signals for environmental stressors (Beketov and Liess, 2008; Belden et al., 2007; Clements and Rohr, 2009). Furthermore, many populations of freshwater macroinvertebrates naturally harbor different kinds of parasites that might bias the outcomes of ecotoxicological studies, but this aspect is often neglected (Goutte and Molbert, 2022; Grabner, 2017; Romero-Blanco and Alonso, 2022). Therefore, it is necessary to consider parasite infections when using field-collected organisms for ecotoxicological studies (Grabner et al., 2023).

The focal species of our study was the freshwater amphipod *Gammarus fossarum* (Koch, 1835). Amphipods are keystone species in aquatic environments due to their widespread distribution, abundance, and diverse ecological roles (Altermatt et al., 2014; Giari et al., 2020). As a detritivore, *G. fossarum* promotes nutrient cycling in aquatic ecosystems and is often considered a model organism for ecotoxicological investigations of environmental contaminants (Consolandi et al., 2019; Fumetti and Blaurock, 2018; Lebrun et al., 2023; Vigneron et al., 2015). Amphipods, including *G. fossarum*, typically host a wide range of micro- and macro-parasites which may affect the hosts' ability to survive, develop, eat, move, reproduce, and react to stressors (Giari et al., 2020; Gismondi et al., 2012a; Grabner and Sures, 2019). Moreover, parasites can manipulate amphipods' behavior resulting in altered movement, phototaxis, or clinging (Bauer et al., 2005; Casalins et al., 2015). The most common amphipod parasites are acanthocephalans and microsporidians (Bojko and Ovcharenko, 2019; Giari et al., 2020; Grabner and Sures, 2019; Prati et al., 2022).

Acanthocephalans have complex lifecycles that involve multiple hosts, including aquatic invertebrates as intermediate hosts (Duarte et al., 2020; Schmidt, 1971; Sures, 2015), and fish or birds as definitive hosts (Giari et al., 2020; Grabner et al., 2023). The acanthocephalan *Polymorphus minutus* (Lühe, 1911; Zeder, 1800) is commonly found in *G. fossarum* (Grabner et al., 2020). Besides altering amphipods' behavior and growth (Grabner et al., 2020; Marriott et al., 1989), *P. minutus* can retain pollutants affecting bioaccumulation patterns in its hosts (Grabner and Sures, 2019; Sures and Nachev, 2022).

Microsporidians are obligate intracellular parasites with complex or simple life cycles (Dunn and Smith, 2001; Stentiford et al., 2013). Recent studies show high microsporidian diversity in amphipods (Bojko and Ovcharenko, 2019; Grabner et al., 2015; Park et al., 2020; Prati et al., 2022). Their virulence depends on their mode of transmission, ranging from high, in horizontally transmitted, to neutral or increased host fitness in vertically transmitted parasites (Dunn and Smith, 2001; Wittner and Weiss, 1999). Similar to acanthocephalans, they might alter host behavior, development, and reproduction (Bojko and Ovcharenko, 2019; Prati et al., 2023; Stentiford et al., 2013). Even though parasites can modulate host physiology, reproduction, and immune responses (Grabner et al., 2023), parasites have mostly been ignored in ecotoxicological investigations (Grabner and Sures, 2019).

Our study provides new insights into the effects of MTZ on field-collected *G. fossarum*, partly being infected by parasites. Besides an acute toxicity experiment, environmentally relevant concentrations of MTZ were used to investigate the sublethal effects of the herbicide while considering the potential bias induced by parasitism. Thereby, this study improves the knowledge of the effects of biotic interactions on toxicologically relevant endpoints. Since herbicides are known to be toxic and can alter behavior (e.g., locomotion, feeding) or biochemical responses even at low concentrations (Bordin et al., 2022; Ferreira Mendes et al., 2023), our first hypothesis is that MTZ exposure will have an acute effect, impacting survival, and sublethal effects, impacting locomotor activity and biochemical responses of *G. fossarum*. Our second hypothesis is that parasites will alter locomotory activity, biochemical responses and chemical stress tolerance in infected *G. fossarum* due to manipulation of host behavior and potential accumulation of pollutants. Lastly, we expect an interactive effect of MTZ and parasites on all biological responses, i.e., mortality, locomotion, and biochemical markers.

## 2. Materials and methods

### 2.1. *Sampling of* Gammarus fossarum

*Gammarus fossarum* individuals were sampled using hand nets and kick sampling in the Rumbach (51°25′27.6''N, 6°54′28.5''E), a right tributary of the River Ruhr in North-Rhine-Westphalia, Germany. The only amphipod present at the site, *G. fossarum,* typically exhibits a high prevalence of the acanthocephalan parasite *P. minutus,* making this location an ideal choice for the current study (Rothe et al., 2022; Zittel et al., 2018). Sampling was conducted between November 2022–March 2023. Individuals collected in November 2022 were used to determine the prevalence of microsporidian infections at the sampling site (November 2022). The *G. fossarum* individuals collected in December 2022 were used to conduct range-finding tests, those collected in January 2023 for MTZ EC50 and LC50 tests, and those collected between January and March 2023 for studying biochemical responses and locomotory activity. All individuals, collected between November 2022–March 2023, that were used for the abovementioned tests were screened for acanthocephalan and microsporidian parasites. *Polymorphus minutus* cystacanths can be easily recognized in the body cavity of living amphipods due to their bright orange coloration (Taraschewski, 2000). During collection, *P. minutus*-infected individuals were visually identified and separated from the uninfected ones. Unfortunately, identification of microsporidian infection requires PCR analysis from host tissue (see below) and is consequently only possible *post mortem* after the experiment.

The collected individuals were kept in river water and transported to the University of Duisburg-Essen (UDE). The individuals were then acclimated for one week prior to the exposure experiments in 10 L containers containing reconstituted water (RW) (OECD, 2007). The containers were oxygenated through air stones, maintaining dissolved oxygen concentration at 8 ± 1 mg/L. The water temperature was kept at 11 ± 1 °C, and the photoperiod was 8: 16 (light: dark). *Gammarus fossarum* individuals were fed with alder leaves collected at the sampling site. The leaves were cleaned, dried, and put in stream-water before feeding.

### 2.2. *Exposure experiment for the investigation of acute immobilization and mortality*

A short-term (96 h) exposure experiment was conducted to determine the effective and lethal concentration of MTZ (Supelco, Merck KGaA, Darmstadt, Germany), purity (% HPLC area) ≥ 98.0 %. The experiment's exposure concentrations were 4 mg/L, 8 mg/L, 15 mg/L, 20 mg/L, and 30 mg/L based on the findings of a preliminary range-finding test. Reconstituted water (OECD, 2007) (RW) without MTZ was used as the control. Each concentration was tested in triplicate, with individuals

divided into *P. minutus* infected (I) and *P. minutus* uninfected (UI) groups. The experiment was conducted in 500-mL glass beakers (VWR International, USA) containing 300 mL of the test solution. Six beakers were set up for each concentration, with three for *P. minutus* infected *G. fossarum* and three for *P. minutus* uninfected individuals, including the control treatment. Each beaker contained ten individuals, totaling 180 infected with *P. minutus* and 180 *P. minutus* uninfected *G. fossarum*. The exposure lasted 96 h at room temperature (17 ± 1 °C) with an 8-h light and 16-h dark cycle, the information on pH and dissolved oxygen is presented in supplementary material (SM) section (Table A3). Experimental beakers were saturated with air through glass pasture pipettes (Duran Wheaton Kimble, Sigma-Aldrich, Merck, Darmstadt, Germany). No food was provided during the exposure period.

Immobility and mortality of *G. fossarum* was observed at 0, 24, 48, 72, and 96 h. Individuals being unable to swim after 30 s following a light agitation of the test vessel were considered immobile, regardless of their ability to move their antennae and legs. Death was defined as the absence of any response when the individual was gently prodded. Exposed *G. fossarum* were inspected three times daily to document any irregularities. A syringe was used to collect a 1 mL water sample at each exposure day for chemical analyses using LC-MS; the subsequent information is presented in SM, Table A1. Dead individuals were promptly collected and preserved in ethanol. The remaining individuals from the exposure experiment were stored in ethanol until their DNA was extracted and screened for microsporidian infections using PCR. However, since in this experiment the individuals were exposed in groups of ten, the observed group effect cannot be correlated to the individual's microsporidian infection status.

### 2.3. Exposure experiment for locomotor activity measurement

The exposure experiment for the activity evaluation was conducted in a climate chamber (temperature: 11.5 ± 0.5 °C, light: dark 8: 16). It comprised six acrylic glass aquaria, each measuring 60 cm × 20 cm and containing 30 L of exposure solution. Each aquarium contained eight Multispecies Freshwater Biomonitor (MFB) chambers developed by LimCo International to investigate the locomotor activity of organisms, and two small circulation pumps (Aquael Circulator 500) for continuous water flow. Each test organism was put in a separate MFB chamber and submerged in the test solution tanks. The MFB chambers have four electrodes: two generate an alternating current electric field, while the other two detect disturbances of the electric field caused by the movements of the test organisms. The individuals were not fed during the exposure experiment. The test solutions were prepared using RW (OECD, 2007). Out of the six aquaria, two were control treatments with only RW, two had 10 μg/L of MTZ in RW, and the other two had 100 μg/L of MTZ in RW. Temperature and dissolved oxygen were measured regularly (Tables A4 and SM). A syringe was used to collect a 1 mL water sample at each exposure day for chemical analyses using LC-MS (Tables A2 and SM). Nets with a mesh size of 63 μm were used to secure both sides of the MFB chamber, allowing light and water to pass through. Frequencies ranging from 0.5 to 8.5 Hz were measured (Gerhardt et al., 1994, 1998). Discrete Fast Fourier Transformation was implemented to process the frequency data. Based on thorough calibration of the signals and concurrent video recordings made during pre-experiments, 1 Hz was chosen as a marker for the locomotor activity in the current study. MFB measurements were recorded every 10 min for a duration of 4 min throughout the exposure period, and subsequently the sum of 24 h activity of each individual was calculated using R v4.3.2 according to Rothe et al. (2022). The overall activity per treatment was determined by calculating the mean combined activity of every individual in the treatment. After exposure, each individual was prepared for molecular analyses for the identification of microsporidian infection and the determination of different biochemical responses.

### 2.4. Sample preparation for molecular analyses

After the exposure period of the locomotor activity experiment, *G. fossarum* were snap-frozen in liquid nitrogen and stored at a temperature of −80 °C for around two weeks until the PCR and biochemical analyses were performed. Prior to biochemical analyses, each *G. fossarum* individual was weighed, cut open, and checked again for *P. minutus* infection. If infected with *P. minutus*, the parasites were removed and counted. Afterwards, the test individual was crushed using a micro-pestle and mixed well in lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA630, 0.1% protease inhibitor cocktail from Sigma Aldrich). Following homogenization, the samples were subjected to centrifugation at 14,000×*g* for a duration of 10 min at 4 °C. After centrifugation, pellets and supernatant aliquots were preserved at −80 °C for successive PCR and biochemical analyses respectively.

### 2.5. Molecular identification of microsporidians

The DNA was extracted from pellets, consisting of the *G. fossarum* tissue, following a modified salt precipitation protocol (Grabner et al., 2015). The extracted DNA was then dissolved in 50 μL TE buffer (Thermo Fisher Scientific, Braunschweig, Germany) and stored at −20 °C for further analyses.

Microsporidians presence in *G. fossarum* was investigated using the universal microsporidian primers V1F (5′-CACCAGGTTGATTCTGCCTGAC-3′) (Zhu et al., 1993) and Micuni3R (5′-ATTACCGCGGMTGCTGGCAC-3′) (Weigand et al., 2016) targeting the small subunit ribosomal RNA (SSU rRNA) gene. Each 20 μL PCR reaction solution contained 10 μL 2x AccuStartTM II PCR ToughMix (Quantabio, USA), 1 μL each primer (0.5 μM), 0.35 μL 50x GelTrack Loading Dye (Quantabio, USA), and 6.65 μL MilliQ water. PCR settings for DNA amplification were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 35 s and 68 °C for 40 s, and a final extension at 68 °C for 5 min. PCR products were then run on 1.5% agarose, and individuals showing a clear ~450 bp band were considered infected.

### 2.6. Biochemical analyses

As a first step, the protein content of the lysate was measured in the supernatant using the Pierce BCA Protein assay kit (Thermo Scientific). A serial dilution of serum bovine albumin (BSA) was used as a standard in a microwell assay, following the instructions provided by the manufacturer. The subsequent test results (2.6.1–2.6.4) were normalized based on the protein composition of the sample.

#### 2.6.1. Catalase activity

Catalase induced reduction of the sample's $H_2O_2$ was measured photometrically (Brand et al., 2019). The sample supernatants were diluted to 5 μg/mL of total protein. The experiment was set up in triplicate using 210 mL of a diluted sample and 90 mL of 100 mM $H_2O_2$. $H_2O_2$ light absorption was measured over time at 25 °C using 96-well UV plates (Greiner Bio-one UV-star) and a microplate reader (TECAN infinite M200). The reaction rate was estimated by measuring absorbance at 240 nm every 15 s for 4 min. To evaluate catalase activity, the reaction curve slope was utilized. A detailed evaluation process is added to the SM (1.1).

#### 2.6.2. Glutathione S-transferase activity

GST activity was measured photometrically. The glutathione-dinitrobenzene conjugate's 340 nm absorbance was measured over time (Brand et al., 2019). To perform the procedure, 5 μL of the 1:10 diluted sample supernatant was incubated with 20 mM L-glutathione and 10 mM CDNB in Dulbecco's phosphate buffer. Measurements were taken on 96-well UV plates using 200 μL triplicates. The microplate reader measured the samples after 10 min incubation at 25 °C. The absorbance was measured every 30 s at 340 nm for 5 min. The reaction

rate in mM/min was determined to be the GST activity. A detailed evaluation process is added to the SM (1.2).

#### 2.6.3. Glycogen and lipid content

The glycogen and lipid content were assessed using the modified procedures of Van Handel (Chen et al., 2015; Handel, 1985). To separate organic and inorganic components, a 2% $Na_2SO_4$ solution and a 1:2 chloroform-methanol solution was used. Following incubation and centrifugation, the supernatant was used to measure lipids, whereas the pellet was used to measure glycogen. To measure glycogen, the pellet was dissolved in 200 μL of deionized water. Standard glucose dilutions were prepared at concentrations of 25, 50, 100, 150, 200, and 400 μg/mL. Next, 100 μL of sample or standard was placed in 15-mL tubes, and 4.9 mL of anthrone reagent (0.2% anthrone in 95% sulfuric acid) was added. The mixture was heated in a 90 °C water bath for 17 min. After 20 s of vortexing, the mixture was chilled on ice. For extinction at 625 nm, 300 μL sub-samples were tested in triplicate using 96-well plates (BRAND™, Fisher Scientific, Germany) and a microwell plate reader. The glycogen standard curve was used to measure glycogen concentration.

Chloroform and methanol were used to establish a cholesterol standard for lipid analysis. Standard concentrations were 0, 25, 50, 100, 200, 400, and 800 μg/mL. Evaporation of 100 μL of each sample and standard in 1.5 mL safe-lock reaction tubes was conducted in a fume hood for 1 h. After adding 200 μL of 95% sulfuric acid, the samples were incubated at 90 °C for 10 min. After cooling on ice, 200 μL of each sample and standard were added to 4.8 mL of vanillin-phosphoric acid reagent. The reagent was made by diluting 300 mg vanillin in 50 mL of hot, deionized water and adding 200 mL of 85% phosphoric acid. After vortexing, 300 μL of sub-samples were analyzed in triplicate at 535 nm using a Tecan Infinite M200 plate reader on the microwell plates. A cholesterol-based standard curve was used to determine the sample lipid content.

#### 2.6.4. Phenoloxidase activity

The PO activity was quantified by spectrophotometry by measuring the conversion of 3,4-dihydroxy-L-phenylalanine (L-DOPA) to dopachrome (Seppälä and Jokela, 2010). For each individual well of a 96-well UV microplate, 20 μL of the sample, 140 μL of $H_2O$ (MilliQ water), 20 μL of PBS buffer (containing NaCl 137 mM, KCl 2.7 mM, $Na_2HPO_4$ 10 mM, $KH_2PO_4$ 1.8 mM), and 20 μl of L-DOPA (Sigma-Aldrich D9628) was used. The samples were measured in triplicates. The experiment was conducted at a temperature of 25 °C and a wavelength of 490 nm. The measurement was taken every 5 min for a duration of 1 h using a Tecan Infinite M200 plate reader. The activity of the PO was analyzed by calculating the slope of the reaction curve. A detailed evaluation process is added to the SM (1.3).

### 2.7. MTZ analysis of water samples

The analysis of MTZ in the water samples of both experiments was conducted using an Agilent 6120 Quadrupole LC-MS equipped with an electrospray ionization (API-ES) source operating in the positive-ion mode. The separation was carried out using an EVO C18 column (Kinetex 5 μm EVO C18 100 Å 100 × 3.0 mm, Phenomenex), and the mobile phase consisted of a gradient of ultrapure water + 0.1% formic acid (A) and acetonitrile (B). The gradient was as follows: 0–2 min (10% B), 3–8 min (45% B), and 12 min (10% B). The volume of the sample injection was 20 μL, and the flow rate was adjusted to 0.5 mL/min. The temperature of the column was set to 31 °C. The instrumental settings for the MS were configured as follows: capillary potential, 3000 V; drying gas (N2) flow rate, 10.5 L/min; gas temperature, 300 °C; nebulizer pressure, 35 psig. The MTZ peak was detected at a m/z of 278, and it had a retention time of 7.4 min.

### 2.8. Statistical analyses

The data on mean overall activity and biochemical analyses (considering both parasite infections and MTZ treatment) were normally distributed according to the Shapiro-Wilk test. Therefore, a two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was employed. MTZ treatment and infection status (infected or uninfected) were used as factors. Biochemical responses in different MTZ treatment groups, without considering parasite infections, were tested for normal distribution using Kolmorogov-Smirnov test. For normally distributed data sets, a one-way ANOVA was carried out. Dunnett's multiple comparison test was applied as a post-hoc test. In case of non-normally distributed data, Kruskal-Wallis test, followed by Dunn's test was performed. The statistical analysis, and the preparation of graphs were carried out using GraphPad Prism version 10.0.2.

## 3. Results

### 3.1. Exposure experiment for the investigation of acute immobilization and mortality

The six tested concentrations of MTZ (ranging from 0 to 30 mg/L) caused no immobility or mortality of the exposed *G. fossarum* infected and uninfected by *P. minutus* during 24 h of exposure (Fig. 1). However, the immobilization rate increased over time (48h, 72h, and 96h) at all tested MTZ concentrations with responses differing slightly between *P. minutus* infected and *P. minutus* uninfected gammarids. All individuals were completely immobilized after 48 h of MTZ exposure at 30 mg/L (Table 1).

For practical reasons, the time-dependent responses of individuals infected or uninfected with microsporidians could not be detected during the exposure period. However, the microsporidian infection was detected at the end of the exposure experiment and it was found that microsporidians were fairly evenly distributed across the test groups (see Tables A5 and SM).

### 3.2. Locomotor activity of G. fossarum under the influence of parasite infection

The overall locomotor activity of *G. fossarum* individuals from the two MTZ treatment groups 10 μg/L and 100 μg/L (Fig. 2) were similar to that of the control group (Fig. 2: A, B). However, significant differences (two-way ANOVA, $F(2, 154) = 5.273$, $P = 0.006$) between individuals' locomotion were observed within each treatment group based on their infection status (Fig. 2: A). *Gammarus fossarum* infected with *P. minutus* only and those coinfected with both *P. minutus* and microsporidians showed increased locomotor activity compared to uninfected individuals (one-way ANOVA, $F = 4.178$, $P = 0.007$). Individuals infected solely with microsporidians showed a statistically not significant (one-way ANOVA, $DF = 43.64$, $P = 0.966$) increase in locomotor activity (Fig. 2: C). MTZ did not have statistically significant (one-way ANOVA, $F = 0.072$, $P = 0.930$) effects on the locomotion of *G. fossarum* (Fig. 2: B). Thus, the increased locomotor activity was driven by *P. minutus* infections (Fig. 2: C).

### 3.3. Biochemical responses of parasite-infected G. fossarum exposed to MTZ

Among the investigated physiological responses, the activities of catalase and PO enzymes increased in *G. fossarum* in the MTZ treatments (Fig. 3) compared to the control treatment (Fig. 3: B, J). The increase in catalase activity was insignificant, whereas that of PO activity was statistically significant (Kruskal-Wallis test, $P = 0.05$). However, individuals infected with microsporidians and *P. minutus* showed slight differences in their enzymatic activities among the treatment groups, including the control. Unlike catalase and PO, the activity of GST

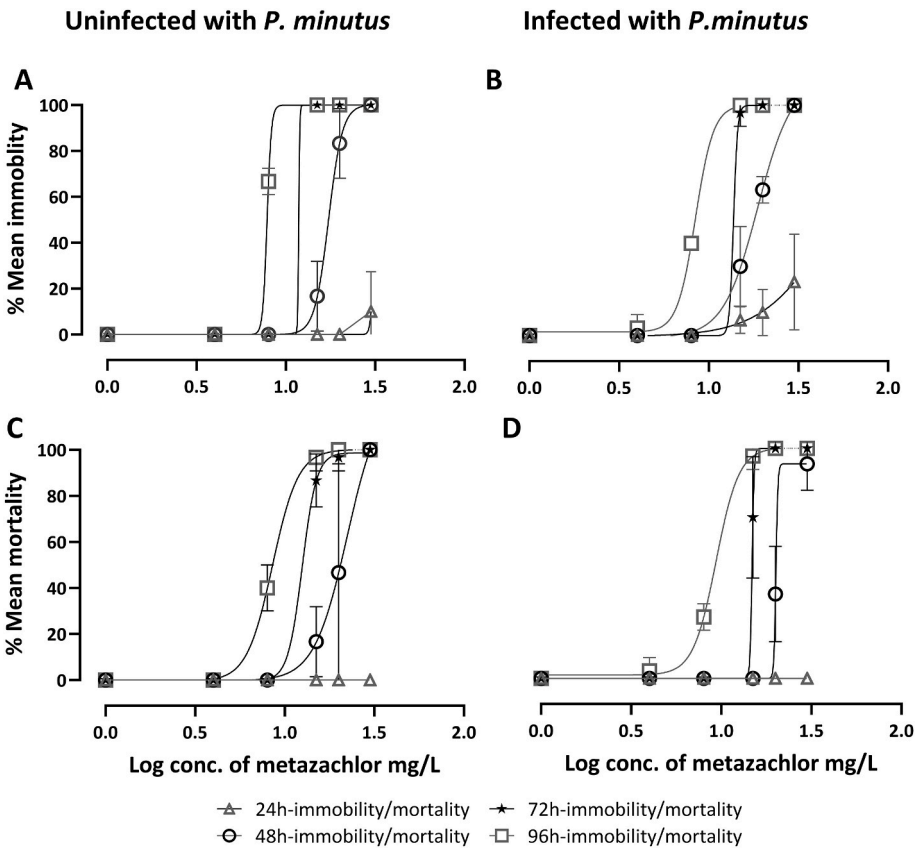


**Fig. 1.** The concentration-response curves of MTZ are shown, with means and standard errors. The respective effects (%) indicating EC50 (A, B) and LC50 (C, D) during the acute (96 h) immobilization test are shown for *G. fossarum* uninfected (A, C), and infected (B, D) with *P. minutus*.

**Table 1**
Summary of the EC50 and LC50 values with 95% confidence intervals (CI) relative to the acute immobilization test of *G. fossarum* infected and uninfected with *P. minutus*.

| Exposure Period | EC50 (mg/L), (CI) Uninfected with *P. minutus* | EC50 (mg/L), (CI) Infected with *P. minutus* | LC50 (mg/L), (CI) Uninfected with *P. minutus* | LC50 (mg/L), (CI) Infected with *P. minutus* |
|---|---|---|---|---|
| 24 h | 32.3[a] | 49.6 (43.9–58.7) | Unstable | Unstable |
| 48 h | 17.3 (17.3–17.3) | 17.7 (16.9–18.7) | 20.0 (18.9–21.3) | 21.3 (20.6–22.1) |
| 72 h | 11.3[a] | 13.8[a] | 12.5 (11.3–13.9) | 14.8[a] |
| 96 h | 7.8[a] | 8.4 (8.0–8.8) | 8.5 (8.4–8.7) | 9.3 (8.8–9.7) |

[a] Indicates time points for which reliable 95% CI for EC50 or LC50 were not calculated due to limited data points.

(Fig. 3_C: two-way ANOVA, $F(2, 115) = 8.094$, $P < 0.001$. Fig. 3_D: one-way ANOVA, $F = 6.488$, $P = 0.003$), and glycogen (Fig. 3_E: two-way ANOVA, $F(2, 114) = 3.492$, $P = 0.034$. Fig. 3_F: one-way ANOVA, $F = 4.345$, $P = 0.015$) declined significantly in individuals exposed to MTZ compared to the control (Fig. 3: D, F). Parasites showed little to no impact on biochemical responses. Neither MTZ treatment nor parasite infection seemed to have a notable impact on the lipid content (Fig. 3: G, H). Overall, environmentally relevant concentrations of MTZ, unlike parasites, impacted the activity of several physiological markers in *G. fossarum*.

## 4. Discussion

This study is the first to evaluate the ecotoxicological effects of the herbicide MTZ on *G. fossarum* while also considering the potential biases introduced by parasitic infections. This is particularly relevant as *G. fossarum* is a keystone species in many aquatic ecosystems, and most ecotoxicological investigations to date tend to neglect parasites (Grabner and Sures, 2019; Zargar et al., 2022).

Our findings on acute effects indicate that MTZ exhibits moderate toxicity towards *G. fossarum*, which supports part of our first hypothesis. Irrespective of parasitic infections, the 96-h LC50 values (8.5 mg/L and 9.3 mg/L) were lower than those found for *Lepomis macrochirus* (15.0 mg/L), and *Cyprinus carpio* (15.0 mg/L), but higher than the 96-h LC50 obtained for *Oncorhynchus mykiss* (4 mg/L) (FAO, 1999), reflecting species-specific differences in physiological responses to MTZ. However, gammarids infected with *P. minutus* were slightly less susceptible to the effects of MTZ than uninfected ones, displaying lower immobility and mortality rates, which partly supports our second hypothesis. A similar outcome was also observed in acanthocephalan-infected *G. roeselii* exposed to the pesticide deltamethrin (Kochmann et al., 2023) and to palladium (Sures and Radszuweit, 2007). Accordingly, acanthocephalans can alter chemical stressor sensitivity of amphipods (Sures et al., 2017). The reduced susceptibility to MTZ in *P. minutus*-infected *G. fossarum* might be due to a higher accumulation of MTZ in the parasite compared to the host tissues. Therefore, infected *G. fossarum* individuals are likely to have a lower concentration of MTZ in the tissues than uninfected ones as was described also for other pollutants (see e.g. Sures et al., 2017, 2023). This suggests an interaction between MTZ and parasites, partly supporting our third hypothesis. However, further investigation on MTZ may provide valuable insights into the complex interaction between parasites and chemical stressors, as well as their effects on hosts.

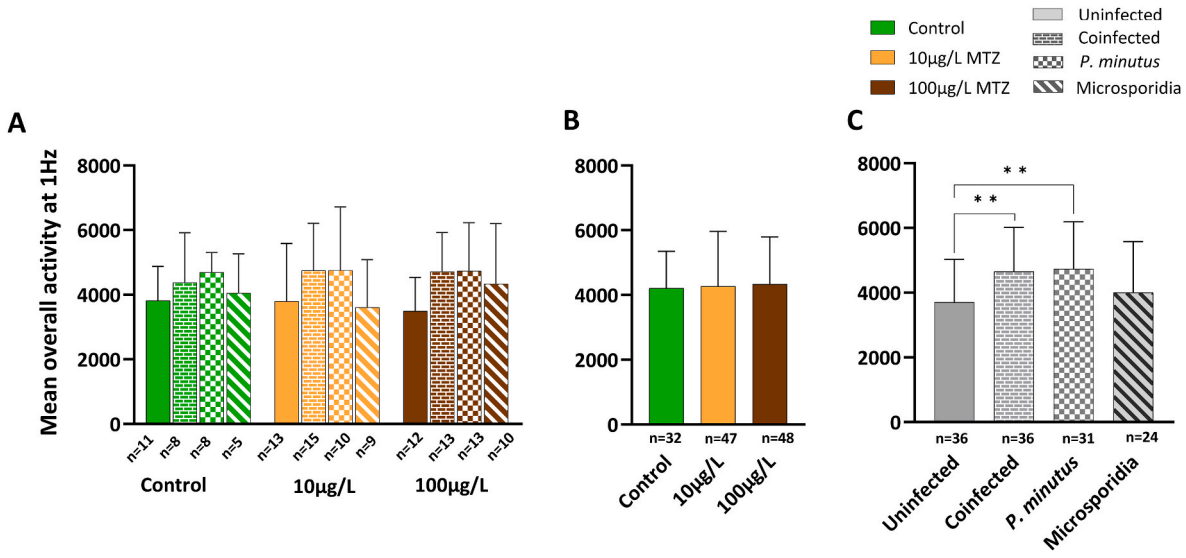


**Fig. 2.** Overall locomotor activity of *G. fossarum* exposed to MTZ (measured at every 10 min for a duration of 4 min over the course of 7 days) and under the influence of parasite infection. Mean overall activity of A: infected and uninfected *G. fossarum* exposed to different concentrations of MTZ, B: different treatment groups irrespective of parasite infection. C: *P. minutus* and microsporidia-infected and uninfected *G. fossarum* without consideration of the MTZ treatment. **: significant differences between groups, $p < 0.002$. A two-way ANOVA followed by Tukey's multiple comparison test was conducted considering MTZ treatment and infection status as factors (A); one-way ANOVA followed by Dunnett's multiple comparison test was conducted to determine significant differences between MTZ treatment groups (B) and between different infected groups (C). The standard deviation is shown by the error bars.

In our sublethal experiments, which focused on environmentally relevant concentrations of MTZ, no significant impact on the locomotor activity of *G. fossarum* was observed. This finding does therefore not support part of our first hypothesis. On the other hand, the observed increase in locomotor activity among the *P. minutus*-infected *G. fossarum*, irrespective of MTZ concentrations, can be attributed to a behavioral alteration, which supports our second hypothesis. A similar response was observed in a recent study where *P. minutus*-infected *G. fossarum* exposed to conventionally, ozone-treated, and river water exhibited significantly higher locomotor activity regardless of exposure solution (Rothe et al., 2022). *Polymorphus minutus* has the ability to modulate the synthesis of neurotransmitters in the host, influencing the serotonergic system and resulting in a phenomenon known as reverse geotaxis (Cornet et al., 2009). Accordingly, *G. fossarum* infected with *P. minutus* swim closer to the water's surface and have altered vertical distribution compared to uninfected ones (Cezilly et al., 2000; Médoc et al., 2006). Such behavioral changes are believed to enhance predation risk, and, thus, facilitate parasite transmission to its definitive bird hosts (Cezilly et al., 2000; Cornet et al., 2009; Médoc et al., 2006). The locomotor activity of gammarids coinfected with microsporidians and *P. minutus* showed no statistically significant difference from the gammarids infected only with the acanthocephalan. This is in contrast with the observation of Haine et al. (2005), who found a weaker activity in coinfected individuals. An explanation for this discrepancy is likely due to the presence of other microsporidian species since some microsporidians are suspected of also altering amphipod behavior (Haine et al., 2005; Prati et al., 2023). Since no interactive effect of parasites and MTZ on locomotion was found, this study could not support part of our third hypothesis.

Infection with acanthocephalans may change the host's physiological homeostasis in the presence of contaminants (Sures, 2008; Ziółkowska and Rokicki, 2003) which can interact with various transcription factors, receptors, and tissues, leading to altered metabolic functions (Khalil et al., 2023; Sures, 2008). Furthermore, if pollutants are present in the environment, the host's detoxification processes can lead to increased metabolic activity (Gismondi et al., 2012b). Since these mechanisms include energy-demanding biochemical reactions, the host's metabolic rate may increase in order to neutralize and detoxify the contaminants (Nassan et al., 2021). Thereby, infection with *P. minutus* may also contribute to a higher metabolic cost for the host, as the host's energy reserve is utilized to maintain homeostasis and cope with parasite infection and pollution (Chen et al., 2015). The combined effect and complex interaction of pollution and parasitism may have chronic effects on the overall fitness of the host due to the host's possible vulnerability to additional environmental stressors (Sures, 2006).

However, part of our first hypothesis is supported by the significant decrease in GST activity and glycogen content, and an increased PO activity in MTZ exposed individuals compared to the control group. The reduced GST activity in *G. fossarum* exposed to high concentrations of MTZ may be indicative of a potential impact on their detoxification processes. GSTs are enzymes involved in the detoxification of xenobiotics, including herbicides, by catalyzing the conjugation of reduced glutathione to electrophilic compounds (Brooks and Mills, 2006). Similar to our investigation, a study on the effects of MTZ on the early life stages of marbled crayfish showed that exposure to environmentally relevant concentrations of MTZ resulted in significantly lower activity of GST, indicating potential metabolic disruption and oxidative stress (Velisek et al., 2020). It has been found that even at high concentrations of herbicides, the GST activity appears low compared to control. For instance, 17.20 mg/L of the herbicide Excel Mera 71 significantly reduced GST activity in the liver of teleostean fish (Samanta et al., 2014). Similarly, decreased GST activities were observed in gills, liver, and muscle of killifish *Jenynsia multidentata* exposed to endosulfan (Ballesteros et al., 2009). Furthermore, a reduction in GST activity was identified in the liver of silver catfish, *Rhamdia quelen,* exposed to Roundup (Menezes et al., 2011). Collectively, these findings indicate that exposure to herbicides can lead to low GST activity, potentially impacting the detoxification processes in exposed organisms.

Similar to GST activity, a significantly reduced glycogen concentration was detected in MTZ-treated *G. fossarum* individuals compared to the control treatment. MTZ inhibits long-chain fatty acid synthesis, which is essential for cell membranes and energy storage (Park et al., 2020). Such disruption may alter metabolic activities including glycogen synthesis (Noack et al., 2004). Glycogen, a branching glucose polymer, serves as a critical energy reserve in organisms, especially in periods of nutritional sufficiency, to provide maintenance energy for cell

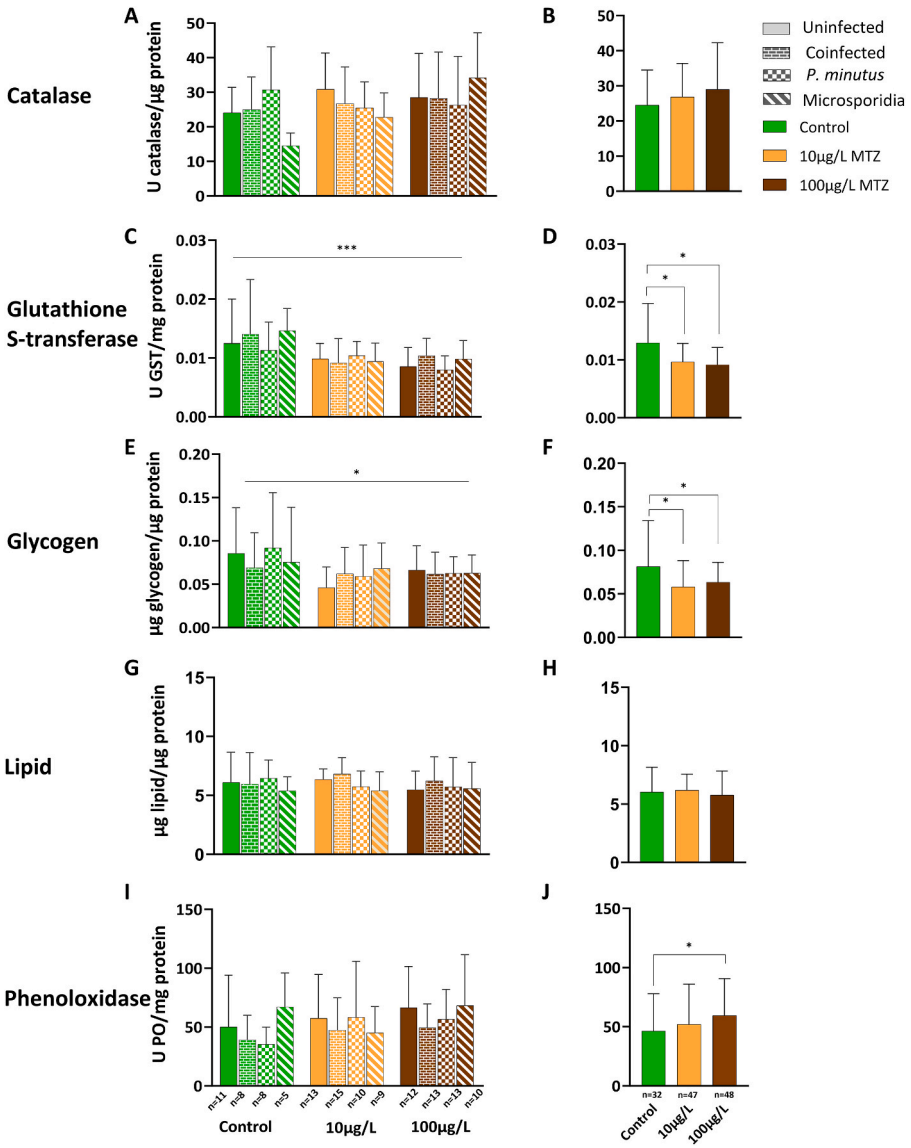


**Fig. 3.** Biochemical responses of *G. fossarum* under the influence of MTZ and parasites. Graphs on the left: infected and uninfected *G. fossarum* exposed to different concentrations of MTZ, graphs on the right: different MTZ treatment groups irrespective of parasite infection. *: significant differences at $p < 0.05$, ***: significant differences at $p < 0.001$. While considering parasite infection and MTZ treatments, a two-way ANOVA followed by Tukey's multiple comparison test was conducted to evaluate the data, with MTZ concentration and infection status as factors. One-way ANOVA followed by Dunnett's multiple comparison test was used to evaluate the data irrespective of parasite infection. The Kruskal-Wallis test followed by Dunn's multiple comparison test was used for the non-normally distributed data. Means are shown, and the error bars represent standard deviation.

integrity, function, and survival in the times of need (Gründel et al., 2012; Roach et al., 2012). MTZ altered crayfish distance and movement speed, which might affect feeding behavior and energy reserves, leading to low glycogen levels (Velisek et al., 2020). An investigation into the acute effects of glyphosate on metabolic and enzymatic parameters of *Rhamdia quelen* revealed a decrease in muscle glycogen stores upon exposure to the herbicide (Glusczak et al., 2007).

In this study, an increased PO activity was observed across the MTZ treatment groups. In response to environmental stressors, some species have shown an increase in PO activity with increased xenobiotic concentrations (Zufelato et al., 2004). For instance, PO activity of the marine blue mussel (*Mytilus edulis*) cells increased ten-fold after preincubation with 0.025% zymosan-supernatant (prepared from yeast-cell-walls, zymosan is used to induce experimental inflammation, and considered a xenobiotic in this context), indicating that the enzyme is activated in reaction to external stimuli (Coles and Pipe, 1994). Treatment with sodium dodecyl sulphate also stimulated PO activity in *Panulirus argus*, a spiny lobster, suggesting a possible reaction to stressors (Perdomo-Morales et al., 2008). Based on these observations, PO activity can be a stress biomarker for ecotoxicological studies employing a variety of organisms, including *G. fossarum*.

In our study, catalase, another enzyme involved in oxidative stress response, did not exhibit any significant changes between the treatment groups when considering parasite infection. However, catalase activity was found to increase with increasing MTZ concentration. Similarly, in a study on the acute effects of glyphosate herbicide on silver catfish, exposure to glyphosate resulted in increased catalase activity in the liver at both concentrations tested (Glusczak et al., 2007). The findings suggest that exposure to chemical stressors such as herbicides can lead to alterations in energy reserves and antioxidant enzyme activities in aquatic organisms.

In contrast to the decreasing GST and glycogen levels in *G. fossarum* with increasing MTZ concentrations, there was no overall trend of the lipid concentrations in the test organisms. Deterioration of lipids is a

possible outcome of oxidative stress and the mechanisms that protect cells from it (Timofeyev et al., 2006). Oxidative stress may account for the lack of discernible patterns in *G. fossarum*'s energy stores as Velisek et al. (2020) found MTZ to potentially impair the oxidative stress defense of marbled crayfish. Therefore, *G. fossarum* might also have depleted its energy reserve in the present study by increasing metabolic rates to protect against oxidative stress. Since the test organisms were not fed while exposed, it is also possible that these outcomes might be the result of food scarcity.

In this study, parasite infection did not influence biochemical responses as assumed, thereby, it could not support part of our second hypothesis. Furthermore, this investigation was unable to corroborate the previous findings indicating that parasite infection can raise lipid and glycogen concentrations in *G. fossarum* exposed to micropollutants (Rothe et al., 2022). However, in the study conducted by Rothe et al. (2022), *G. fossarum* were exposed to treated wastewater instead of a single chemical substance. Therefore, the different chemical composition of the water used and their possible interactions with the host might explain such discrepancy. However, the lack of clear indication of parasites' interaction with MTZ in modulating biochemical responses in this study could not support part of our third hypothesis.

## 5. Conclusion

In our study, the acanthocephalan parasite *P. minutus* notably affected *G. fossarum*'s locomotor activity, suggesting that parasites can alter host responses to environmental stressors. MTZ, on the other hand, induced notable physiological responses in *G. fossarum* even at environmentally relevant concentrations. This highlights the need for further investigation into sublethal endpoints and the potential stressor role of MTZ. The investigations underline the role of parasites as potential stressors, however, their interactions with contaminants are complex and difficult to unravel. Nevertheless, our findings collectively suggest that both parasites and MTZ contribute to the overall environmental stress experienced by aquatic organisms including non-target species, e.g. *G. fossarum* in natural ecosystems. Ignoring either aspect in ecotoxicological studies can lead to biased outcomes. In future ecotoxicological studies, it is crucial to account for parasites and use environmentally relevant contaminant concentrations to more accurately assess the impact on non-target species in natural ecosystems.

## Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, scienceOS and QuillBot were used as AI-assisted technologies to search for relevant references, improve the readability and language of the manuscript, paraphrase, check grammar and spelling. After using the tools, the authors reviewed and edited the content as needed taking full responsibility for the content of the published article.

## CRediT authorship contribution statement

**Nazmun Nahar:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ipsita Sarkar:** Writing – review & editing, Visualization, Formal analysis, Data curation. **Sebastian Prati:** Writing – review & editing, Visualization, Validation, Methodology. **Louisa E. Rothe:** Writing – review & editing, Visualization, Validation, Methodology. **Daniel Grabner:** Writing – review & editing, Visualization, Validation, Methodology. **Sonja Zimmermann:** Writing – review & editing, Visualization, Validation, Methodology. **Anam Asghar:** Writing – review & editing, Visualization, Validation, Methodology. **Torsten C. Schmidt:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Conceptualization. **Bernd Sures:** Writing – review & editing, Visualization, 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.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.125413.

## Data availability

Data will be made available on request.

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