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Effects of environmentally relevant concentrations of microplastics on amphipods

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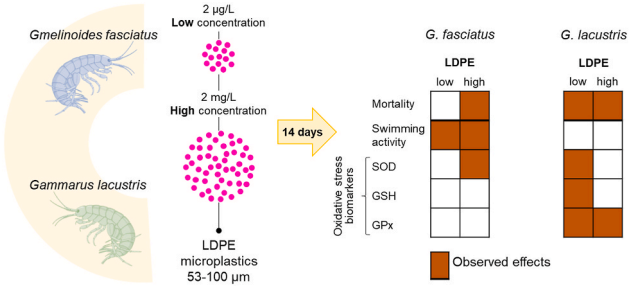
HIGHLIGHTS

- The effects of LDPE microplastics (MP) on *Gammarus fasciatus* and *Gmelinoides lacustris* were studied.
- LDPE MP triggered different responses on *G. fasciatus* and *G. lacustris*.
- *G. fasciatus* was more sensitive towards swimming-related effects.
- Exposure to 2 μg/L LDPE MP induced oxidative stress in *G. lacustris*.
- 2 mg/L LDPE MP increased mortality of both amphipods.

GRAPHICAL ABSTRACT



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ABSTRACT

Lack of microplastics (MP) toxicity studies involving environmentally relevant concentrations and exposure times is concerning. Here we analyzed the potential adverse effects of low density polyethylene (LDPE) MP at environmentally relevant concentration in sub-chronic exposure to two amphipods *Gmelinoides fasciatus* and *Gammarus lacustris*, species that naturally compete with each other for their habitats. 14-day exposure to 2 μg/L (8 particles/L corresponding to low exposure) and 2 mg/L (~8400 particles/L, corresponding to high exposure) of 53–100 μm LDPE MP were used to assess ingestion and egestion of MP, evaluate its effects on amphipod mortality, swimming ability and oxidative stress level. Both amphipod species were effectively ingesting and egesting LDPE MP. On the average, 0.8 and 2.5 MP particles were identified in the intestines of each amphipod exposed to 2 μg/L and 2 mg/L LDPE MP, respectively. Therefore, intestinal MP after 14-day exposure did not fully reflect the differences in LDPE MP exposure concentrations. Increased mortality of both amphipods was observed at 2 mg/L LDPE MP and in case of *G. lacustris* also at 2 μg/L exposure. The effect of LDPE on swimming

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activity was observed only in case of *G. fasciatus*. Oxidative stress marker enzymes SOD, GPx and reduced glutathione GSH varied according to amphipod species and LDPE MP concentration. In general *G. lacustris* was more sensitive towards LDPE MP induced oxidative stress. Overall, the results suggested that in MP polluted environments, *G. lacustris* may lose its already naturally low competitiveness and become overcompeted by other more resistant species. The fact that in the sub-chronic foodborne exposure to environmentally relevant and higher LDPE MP concentrations all the observed toxicological endpoints were affected refers to the potential of MP to affect and disrupt aquatic communities in the longer perspective.

1. Introduction

Microplastics (MP) with dimensions less than 5 mm, formed either by fragmentation of larger plastic pieces (Eerkes-Medrano et al., 2015) or intentionally produced by industry for a specific purpose (Carr et al., 2016), is a subject of increasing environmental concern. Currently, there are hundreds of different types of plastic (Andrady and Neal, 2009), with 90% of global production corresponding to polyethylene (PE), polypropylene, polystyrene, polyvinyl chloride and polyethylene terephthalate (Geyer et al., 2017). Of these, all have already been identified in the environment in MP size (Horton et al., 2017; Klein et al., 2015).

Most identified MP particle morphologies are irregular shapes (Vianello et al., 2013) with sizes between 50 and 630 μm (Klein et al., 2015; Laermanns et al., 2021). However, most of the MP studies involve regularly shaped particles, such as microspheres (Burns and Boxall, 2018; Phuong et al., 2016), thus distancing the results from reality (Connors et al., 2017; Kooi and Koelmans, 2019). Analogously, the size of MP particles is often not corresponding to their environmental size range. Many studies have been performed using particles smaller than 50 μm, showing that those exhibit toxicity (Fu et al., 2019; Jeong et al., 2016). For this reason, it is necessary to study the size ranges that are also frequently found in the environment.

One additional shortcoming of the current MP studies in aquatic environments is the use of unrealistically high exposure concentrations (Koelmans et al., 2019). Vast majority of studies are done with MP particle concentrations between 50 and 10,000 particles per mL (Capolupo et al., 2018; Phuong et al., 2016), which significantly exceed the measured MP concentrations in the natural environment. In natural waters, the concentrations of MP have been shown to range between tens and hundreds of particles per liter (Alam et al., 2019; Ding et al., 2019; Kreitsberg et al., 2021; Leslie et al., 2017; Mani et al., 2015; Schmidt et al., 2018; Scircle et al., 2020; Watkins et al., 2019; Zhang et al., 2017; Zhu et al., 2019) and in general, there is a lack of studies involving such concentrations as well as longer exposure times (Connors et al., 2017; Phuong et al., 2016).

Another shortcoming of the current research on MP is its emphasis on marine environment (Meng et al., 2020; Wang et al., 2021) whereas MP impacts on freshwater ecosystems are far less studied. However, given the importance of freshwater habitats for the maintenance of biodiversity, it is essential to carry out more studies to reveal the effects of MP also to freshwater biota (Castro-Castellon et al., 2022). So far, the studies of MP effects on freshwater organisms indicate that MP are not acutely toxic and sub-toxic long-term effects should be looked for instead. One of the marker organisms for freshwater ecosystems are crustaceans. In this study we specifically focused on two amphipods whose responses to MP exposure have only been superficially studied. These benthic detritivorous amphipods *Gmelinoides fasciatus* and *Gammarus lacustris* were chosen for their environmental relevance and competitive nature. Competition mechanisms and predation on *G. lacustris* by *G. fasciatus* is not entirely known, but likely it occurs as a result of the faster reproduction rate of *G. fasciatus* (Berezina, 2004, 2005). These amphipods play a key role in the food web (Lubyaga et al., 2020; Mateos-Cárdenas et al., 2021) and are adaptable to adverse environmental conditions (Berezina, 2009; Panov and Berezina, 2002; Vereshchagina et al., 2021), however research on the impact of MP-related stress for *G. lacustris* and *G. fasciatus* is almost lacking. Studies carried out with other amphipods have shown that MP induces disturbed feeding behavior (Yardy and Callaghan, 2020), growth reduction (Redondo-Hasselerharm et al., 2018), reproduction and egestion (Au et al., 2015). In general, the most frequently used toxicity endpoints for MP-exposed amphipods have been behavioral changes (Bartonitz et al., 2020), mortality (Gerhardt, 2020) and ingestion and/or egestion (Blarer and Burkhardt-Holm, 2016; Iannilli et al., 2019; Gerhardt, 2020; Mateos-Cárdenas et al., 2021) while for example oxidative stress and gene expression have not been studied thus far.

To address the shortcomings of the current studies, we analyzed the behavioral and biochemical responses of two amphipod species, *G. fasciatus* and *G. lacustris* upon their sub-chronic exposure (>10 days) to an environmentally relevant concentration of (LDPE) MP. The tested LDPE MP were irregular shaped and corresponded to the size of MP recorded in the environment. LDPE MP was tested at two concentrations; one of which was considered environmentally relevant (2 μg/L, low concentration) and the other that was similar to the studies that have used high exposure concentrations (2 mg/L). Ingestion of MPs and following mortality of the two amphipod species were registered. According to previous studies indicating low acute toxicity of MP, also sub-toxic endpoints such as induction of oxidative stress enzymes and changes in swimming activity, were recorded. Our hypothesis was that environmentally relevant MP concentrations may induce oxidative stress and behavioral changes in amphipods at sub-chronic exposures. Considering the dominant nature and greater tolerance to unfavorable environmental conditions of *G. fasciatus*, we hypothesized that *G. lacustris* may be more vulnerable to environments with anthropogenic disturbances, including to the MP contamination.

2. Material and methods

All the chemicals used in the study were of analytical grade. Ultrapure water was used throughout the study.

2.1. Study organisms

The study organisms were small freshwater amphipods *G. fasciatus* (Stebbing, 1899) and *G. lacustris* Sars, 1863. *G. fasciatus* and *G. lacustris* were collected from Estonian freshwater lakes: *G. fasciatus* from Lake Peipus (58°14′00.5″N, 27°28′37.1″E), and *G. lacustris* from Lake Võrtsjärv (58°12′48.9″N, 26°06′36.7″E). Adult specimens were collected between June and September 2021 from the shallow water using a kick-net with a mesh size of 0.05 mm. The animals were acclimatized to experimental conditions for a week (14 ± 1 °C; 98 ± 2% dissolved oxygen; 8 ± 0.5 pH; 0.7 ± 0.2 Nephelometric Turbidity Units, measured with a YSI-PRO DSS Multiparameter analyzer). After the acclimatization period, the animals were gently placed in the test tanks for 24 h before exposure in the absence of food. Prior to the experiment, a visual analysis was made to observe the presence of dead or debilitated animals, which were then removed from the tanks.

2.2. LDPE microplastics preparation and characterization

Our study involved microparticles of LDPE polymer that is one of the most produced polymers in the world. MP particles were provided by Icopolymers (ICO Polymers, a division of A. Schulman, Allentown, PA,

USA) in the form of powder. The chemical composition of this material has been confirmed by Kokalj et al. (2021). LDPE powder was fractionated through a consecutive series of stainless-steel sieves (500, 250, 100 and 53 μm) (Godoy et al., 2019; Stock et al., 2019) until LDPE particles of 53–100 μm were obtained. For better visualization the sieved particles were stained using Nile Red fluorescent dye (99% pure, ACROS Organics™, Geel, Belgium) (staining procedure described in SI). Olympus BX51 Fluorescence Microscope with a 480/40 nm emission filter was used to ascertain the MP size range on 300 random particles.

For particle concentration, 20 and 30 μg of MP particles were counted under a Nikon SMZ1500 Stereomicroscope. The process was performed in triplicate. For every 20 μg of LDPE MP we found 83.6 ± 3 particles and in 30 μg we found 126.7 ± 2 particles. To calculate how many MP particles were in our exposure concentrations (2 μg/L corresponding to 14 μg per tank and 2 mg/L corresponding to 14 mg per tank), conversion calculation was used. The concentration of MP particles in tanks expressed as particles in tank or per liter is shown in Table 1. Exposure concentration of 2 μg/L corresponding to 8 LDPE particles/L or 59 particles per tank was considered environmentally relevant based on earlier reviews showing the presence of up to 34 MP/L in natural waters (Dusaucy et al., 2021). 2 mg LDPE/L or ~8400 LDPE particles/L was used as high concentration, to be comparable with most of the previous MP toxicity studies with amphipods (Scherer et al., 2017; Weber et al., 2018). In parallel to LDPE MP, natural silica particles (SiO_2, white quartz −50 + 70 mesh, Sigma Aldrich) that were used in further assays as natural particle controls, were passed through the same sieve system as the MP to ensure the same size range of 53–100 μm.

2.3. Exposure of amphipods to LDPE microplastics

2.3.1. Preparation of LDPE microplastics-spiked food

For preparation of LDPE MP-spiked food, 14 μg (corresponding to 2 μg/L exposure) or 14 mg (corresponding to 2 mg/L exposure) LDPE MP was mixed with 70 mg of flaked fish food (JBL NovoBel), which was the portion of the food that was placed in one tank. MP and food were mixed until homogeneity, then 250 μL distilled water was added, the mixture was stirred again and then dried in an oven at 60 °C for 24 h to form a pellet. This pellet was cut to smaller pieces and in a porcelain container placed on the bottom of a tank. Analogously to LDPE MP, the sieved SiO_2 were mixed with 70 mg of fish food at 14 μg (corresponding to 2 μg/L exposure) or 14 mg (corresponding to 2 mg/L exposure) and dried before introduction to the tanks as described above.

2.3.2. Experimental design for exposure of test animals to LDPE microplastics and control compounds

LDPE MP were administered to amphipods via foodborne exposure. 70 mg of solid food pellets (see above) were placed in each tank inside porcelain containers (Fig. S1). Altogether 18 10-liter tanks filled with 7 L of preconditioned water were used per test animal, three tanks for every five exposure conditions (Table 1) and a non-exposed control. In case of LDPE MP exposure, the food pellets contained either 2 μg/L (14 μg per tank) or 2 mg/L (14 mg per tank) LDPE and in case of SiO_2 exposure, the food pellets contained 2 μg/L (14 μg per tank) or 2 mg/L (14 mg per tank) SiO_2. Into tanks with paraquat (Sigma-Aldrich Chemie GmbH), used as a positive control for oxidative stress (Lascano et al., 2012), 70 mg of pure food pellets were added. Into every tank with 7 L of water, 70 individuals of *G. fasciatus* or *G. lacustris* were placed. All the tanks were kept in controlled conditions at 14 ± 1°C and a 16:8 (light:dark) photoperiod and constant aeration (Fig. S2). The testing lasted 14 days. The water parameters of each tank (temperature, dissolved oxygen, conductivity, pH and turbidity) were recorded on days 1, 7 and 14 (Table S1).

Once a week, 50% of the water was exchanged, old food was removed, excrements were collected for later analysis of LDPE MP egestion and fresh spiked or non-spiked food was supplied. In order to avoid potential cross-contamination with background MP, all materials and equipment that had direct contact with the samples were made of glass, porcelain or metal. Besides that, cotton or wool based clothes were worn whenever possible during all procedures. In addition, samples of organisms from the control groups (negative control, natural particle control and positive control) were also analyzed following the same protocols to verify the ingestion and egestion of MP.

2.4. Effects of environmentally relevant concentrations of microplastics on amphipods

2.4.1. LDPE microplastics ingestion and egestion by amphipods

Ingestion of MP was studied by analyzing the intestinal/gut contents of five *G. fasciatus* and *G. lacustris* animals from each tank on days 1 and 14. Once removed from the tanks, the animals were placed on glass slides to have their intestines removed under a Nikon SMZ1500 Stereomicroscope. The whole intestines were then analyzed under fluorescence microscope Olympus BX51 Fluorescence Microscope (480 nm LED emission) so that the fluorescent particles could be visualized and counted on glass slides. Egestion of MP was quantified by the presence of particles in the excrements on the bottom of the tanks. 50 mL of water with feces was collected in glass bottles on days 7 and 14. Organic matter in the samples was digested with 30% KOH for 48 h at 40°C. The contents were then vacuum filtered and retained on a 25 mm Whatman fiberglass membrane with pore size of 0.7 μm. For counting the particles, the filters were analyzed under fluorescence microscope as described above. For particle counting, a manual counter was used and particles were photographed using Olympus XM10 camera and Olympus cellSens Standard imaging software.

2.4.2. Analysis of mortality of amphipods

During exposure, the tanks were monitored for amphipods' mortality on days 7 and 14. Mortality was expressed as a percentage of the initial number of organisms in the experiment. Due to the cannibalistic nature of the studied species, all the dead animals were immediately removed from the tanks as soon as they were observed.

2.4.3. Analysis of swimming activity of amphipods

The change of behavior in response to 14 days of LDPE MP exposure was recorded by filming the swimming activity of the amphipods at the beginning of the experiment and after 14 days of exposure. For filming, three amphipods were placed into an 11 cm diameter Petri dish filled with water from the tank and left to acclimatize for 2–3 min. Swimming was filmed for 2 min and the films were analyzed using the program LoliTrack (Loligo Systems, Denmark) for the total swimming time of

Table 1
The different exposures used in the study. For LDPE microplastics (MP), the total number of particles in the tank (p), and particles/L (p/L) are shown. These data are average for n = 3 measurements.

Test compound	Concentration	w/L	w/tank	p	p/L	Purpose
LDPE MP	Low	2 μg	14 μg	59	8	Environmentally relevant concentration of MP
	High	2 mg	14 mg	59000	~8400	High exposure concentration of MP
SiO_2	Low	2 μg	14 μg	n.a.	n.a.	Natural particle control for low exposure
	High	2 mg	14 mg	n.a.	n.a.	Natural particle control for high exposure
Paraquat		1 μg	7 μg			Positive control for oxidative stress

animals (in %). Three animals were filmed from each 14-day treatment (altogether swimming activity of 316 animals was analyzed: three individuals were collected and filmed twice per each tank resulting in 18 from each treatment). A baseline swimming activity was established for both, *G. fasciatus* and *G. lacustris* prior to the start of the experiment.

2.4.4. Analysis of oxidative stress markers in amphipods

Oxidative stress markers superoxide dismutase (SOD), glutathione (GSH) and glutathione peroxidase (GPx) were measured after 14 days of exposure to LDPE MP and compared with the levels before the experiment. For that, 10 animals were removed from each tank and stored at −70°C in the KPE buffer (pH 7.5) until the analysis. Immediately before the analysis, organisms were put in a tissue homogenizer device (Bullet Blender STORM 5) at speed 10 for 10 min. The details of each assay are given in SI. For the analysis of SOD and GPx enzymes as well as for total protein analysis, the homogenized samples were centrifuged (15 min, 3000 rpm, 4°C). The supernatant was transferred to new tubes and used for absorbance measurement along with the respective reagents (SI). Measurement of SOD followed the methods described by Urvik et al. (2016) while GSH and GPx followed Vives-Bauza et al. (2007) with modification to better suit our samples (SI). For GSH quantification, an aliquot of the homogenized tissue sample was mixed with trichloroacetic acid (TCA) in a 1:1 ratio and vortexed 3x every 5 min. Then, the sample was centrifuged (15 min, 3000 rpm, 4°C). The supernatant was transferred to new tubes and used for absorbance measurement along with the respective reagents (SI). SOD, GPx and GSH results were measured by spectrophotometric absorbance using Synergy 2 plate reader and Gen 5 software (BioTek). All the oxidative stress marker assays were performed in a random order. To prevent enzymatic content variations due to organisms' size differences, the values for all enzymatic markers were normalized to total protein content (O'rourke et al., 2019). The total protein content was measured from homogenized samples using Bio-Rad protein assay which is based on the Bradford dye-binding method (SI).

2.5. Statistical analysis of the data

The endpoints of mortality, MP ingestion, and egestion were compared using analysis of variance (ANOVA) method to determine if there were significant effects of MP exposure time and concentration. ANOVA was followed by a series of contrasts evaluated with t-tests to determine mean differences between treatments exposed to LDPE MP and those that were not exposed to either MP at the two chosen concentrations. These calculations were performed in RStudio v 1.3.1093 (R Core Team, 2020). All analyzes were performed in triplicate and statistical significance has been accepted at $p < 0.05$ level.

3. Results and discussion

3.1. Characteristics of LDPE microplastics

LDPE MP that was fractionated from the commercial LDPE powder to 53–100 μm size and fluorescently stained with Nile Red was viewed under fluorescence microscope (Fig. 1). Irregular morphology and expected size range of the particles was confirmed. The measurement of 300 particles under fluorescence microscope showed that the maximum size of LDPE MP was 190 μm and the minimum size 41 μm, while the average diameter was 81.03 ± 20.32 μm.

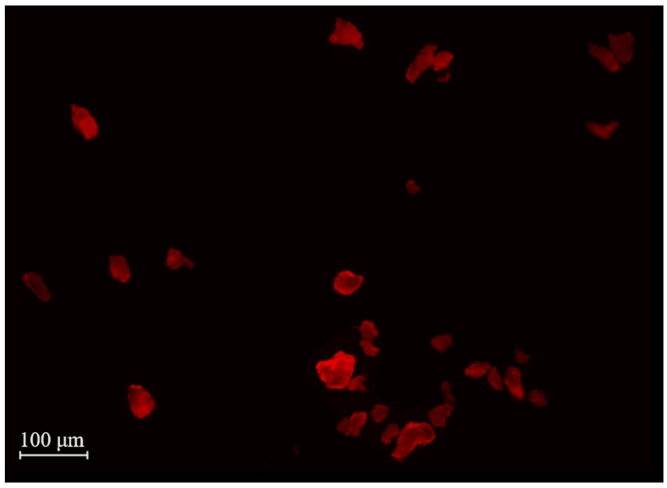

Fig. 1. Visualization of Nile Red stained LDPE MP particles under fluorescence microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Microplastics ingestion and egestion by amphipods

As a rule, one of the prerequisites for toxicity of particulate compounds is their ingestion. Therefore, prior to toxicological tests ingestion-egestion balance of LDPE MP in *G. fasciatus* and *G. lacustris* during 14 days of exposure was studied. For ingestion analysis, MP was counted in the guts and for egestion analysis, MP was counted in excreted feces. Analyzes performed on all the treatments on day 1 (n = 5) showed that the guts were MP free.

The results showed that the difference between MP in the guts of low and high exposures was not corresponding to the differences in LDPE MP exposure concentrations. While at low concentration, the average number of MP in the gut of one *G. fasciatus* or *G. lacustris* after 14 days was 0.8, then at high concentration this number was 2.5 (Table 2). However, there was a clear difference between egested LDPE MP in low and high exposures as in the latter, the number of egested particles was 30–100 times higher than in the former. Representative images of LDPE MP in the guts and excrements of *G. fasciatus* and *G. lacustris* are given in Fig. 2. No visible changes of the MP were identified during the 14-day exposure. It is important to highlight that some of the particles were detached from the food pellet and, due to its physicochemical characteristics, moved to the water surface. Thus, these MP were no longer available for ingestion, which may have also influenced the number of particles ingested.

Table 2
Ingestion-egestion balance of low-density polyethylene microplastics (LDPE MP). The number of LDPE MP particles present in the intestines of each *Gmelinoides fasciatus* (n = 29) and *Gammarus lacustris* (n = 10) after 14 days of exposure to low or high MP concentration. The number of egested MP particles indicates the mean number of MP per tank (50 mL of excrement sample) after 7 and 14 days of exposure to low and high LDPE MP. Data are shown with ±SD. n.a. not available.

		Exposure			
		LDPE MP low 2 μg/L		LDPE MP high 2 mg/L	
		7 days	14 days	7 days	14 days
G. fasciatus	Intestinal MP	n.a.	0.8 ± 1.2	n.a	2.5 ± 1.6
	Egested MP	1.3 ± 1.5	1.0 ± 1.0	131.7 ± 9.0	176.7 ± 14.6
G. lacustris	Intestinal MP	n.a.	0.8 ± 0.8	n.a	2.6 ± 1.7
	Egested MP	2.7 ± 1.1	5.0 ± 4.0	124.0 ± 12.5	146.0 ± 15.5

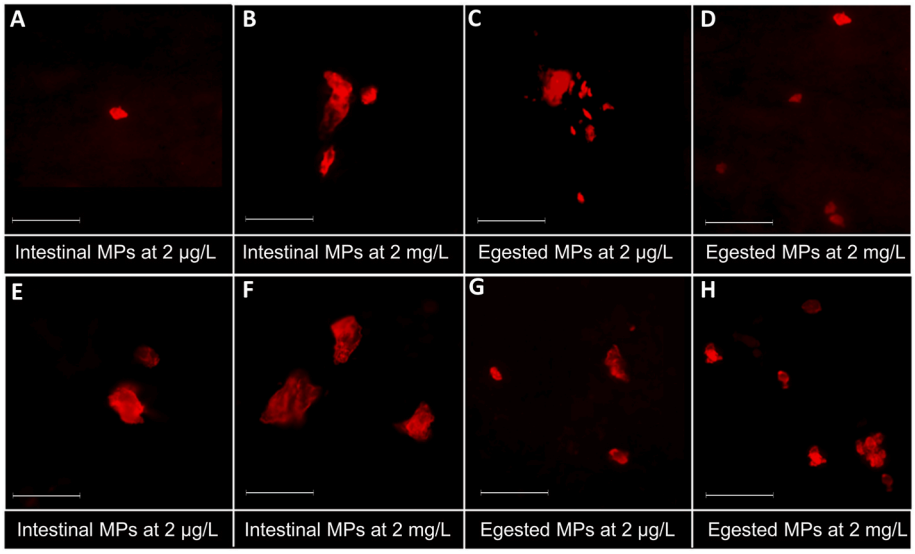

Fig. 2. Ingested fluorescent LDPE microplastics (MP) recovered from the guts of *Gmelinoides fasciatus* (A–B) or *Gammarus lacustris* (E–F) and egested LDPE MP retrieved from excrements (C-D of *G. fasciatus*; G-H of *G. lacustris*) from low LDPE MP exposures (2 µg/L) and high (2 mg/L) exposures. Scale bars represent 100 µm. All images show representative views of the specified samples.

Earlier studies have demonstrated that amphipods are effective in ingesting particulate matter, including anthropogenic polymers (Driscoll et al., 2021; Yardy and Callaghan, 2020) e.g. polystyrene (PS) beads, polyester fibers, polyethylene terephthalate (PET) fragments and polymethylmethacrylate (PMMA) (Mateos-Cárdenas et al., 2021; Scherer et al., 2017; Setälä et al., 2016; Straub et al., 2017; Weber et al., 2018). Here we showed that both, *G. fasciatus* as well as *G. lacustris* were able to ingest 52–100 µm fragments of LDPE MP. For *G. fasciatus*, ~100 µm MP has been shown to be the most preferred fraction among 250 µm > MP < 50 µm thus this species is hypothesized to play a significant role in transport and trophic transfer of ~100 µm MP (Kalinkina et al., 2022). Although we examined the intestines of *G. fasciatus* and *G. lacustris* only after 14 days of exposure, we strongly believe that MP ingestion takes place already from the beginning of the exposure. Our data showing the ingestion of plastic particles even in low LDPE MP treatment indicated that *G. fasciatus* and *G. lacustris* are capable of ingesting MP also under existing MP pollution conditions. Such intake could become one of the entry points of MP into the food chain, especially since gammarids have been shown not to avoid MP-contaminated food (Mateos-Cárdenas et al., 2022). According to our results, there were no significant differences between the intake of LDPE MP by *G. fasciatus* and *G. lacustris* (Table 2; $p > 0.05$). Such a result could be expected considering the relatively similar size of those animals (*G. lacustris* ranges from 8.0 to 12 mm in length and *G. fasciatus* ranges from 7.0 to 10 mm in length) and the ability of *G. fasciatus* to compensate its slightly smaller size with larger appetite (Berezina, 2009).

Our results on the number of intestinal MP showed that ingestion of LDPE MP at high exposure concentration was about 3-fold higher than at low exposure concentration. Dose dependent ingestion of MP has also been observed in previous studies (Weber et al., 2018). However, in the current study the effect of exposure concentration on intestinal MP was not as high as it could have been expected based on the difference between low and high exposures (Table 1). These results suggest that the animals have an intake limit, so even if the exposure concentration was increased, the number of intestinal MP would probably remain constant. Considering the relatively low number of MP particles remaining in intestines we suggest that LDPE MP residence time in amphipod guts is relatively short and the particles pass through the digestive system quickly. However, incomplete LDPE MP egestion may lead to trophic transfer. Finally, considering the active passing of MP particles through the intestines and their presence in feces, re-exposure of amphipods to MP may take place also via fecal pellets that constitute an important share of macroinvertebrates diet (Joyce et al., 2007).

Interestingly, after egestion by the biota, MP can change the density of feces pellets (Pérez-Guevara et al., 2021). When LDPE MP were egested by *Calanus helgolandicus*, density of fecal pellets was decreased, resulting in greater particle float, while polyethylene terephthalate MP increased fecal density (Coppock et al., 2019). Considering the results of this study, we conclude that, depending on the type of polymer, ingestion/egestion of MP can constitute an important route of exposure for certain species, since MP may now be more available in the water column or in the sediment.

3.3. Mortality of amphipods due to LDPE microplastics

Survival of *G. fasciatus* and *G. lacustris* upon exposure to low (2 µg/L) and high (2 mg/L) LDPE MP concentrations, positive control (1 µg paraquat/L) and the natural particle control (SiO_2), is shown in Fig. 3. Our results showed 10–23% mortality of amphipods, including in no exposure conditions. This background mortality was likely caused by different water parameters compared to the natural environment of the animals despite the week-long acclimatization after catchment and 24-h acclimatization in the test tanks. However, this high mortality could be also a result of the cannibalistic behavior of the two amphipods used as discussed by Vereshchagina et al. (2021). Compared with no exposure conditions, high LDPE MP exposure resulted in small but significant increase in mortality of *G. lacustris* both, upon 7 and 14 days of exposure and of *G. fasciatus* upon 14 days of exposure (Fig. 3 B, C, D). Low LDPE MP exposure did not induce significant mortality other than in case of *G. lacustris* upon 14 days of exposure (Fig. 3 C). SiO_2 particles did not induce mortality but on the contrary, low concentration of SiO_2 reduced background mortality. The oxidative stress control compound paraquat induced slight but significant mortality of *G. fasciatus* upon 14 days of exposure and of *G. lacustris* upon 7 days of exposure.

Currently, there is no consensus regarding increased mortality of aquatic organisms due to MP exposure. While some studies indicate that exposure to MP does not induce mortality of crustaceans *Echinogammarus marinus, Gammarus duebeni, Gammarus pulex and Hyalella azteca* (Bruck and Ford, 2018; Mateos-Cárdenas et al., 2019; Redondo-Hasselerharm et al., 2018; Weber et al., 2018), others suggest the opposite for the same animal group (Au et al., 2015; Gerhardt, 2020). The few MP studies on amphipods are contradicting as well. For

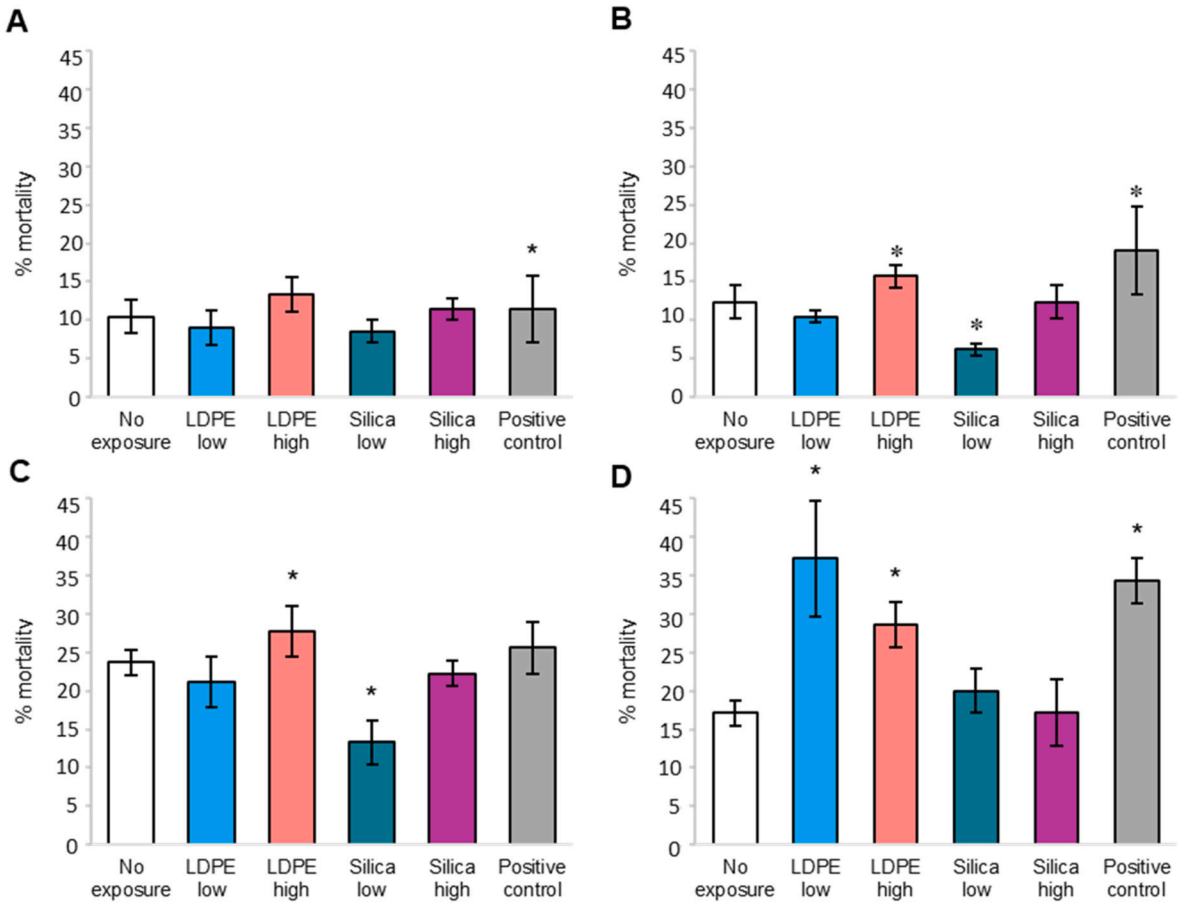

Fig. 3. Mortality of amphipods *Gmelinoides fasciatus* (A: after 7 days; B: after 14 days) and *Gammarus lacustris* (C: after 7 days; D: after 14 days) after exposure to LDPE microplastics (MP), positive control paraquat and to natural particles of SiO_2 (silica). Data of three aquariums are shown. * represents a statistically significant difference ($p < 0.05$) in mortality between the specific treatment and no exposure condition.

example, for *Gammarus fossarum,* ingestion of environmental MP (Driscoll et al., 2021) and subsequent reduction in assimilation efficiency (Straub et al., 2017) has been shown. However, Bartonitz et al. (2020) suggested that exposure to MP is not inducing toxic effects in *Gammarus roeseli* and similar has been suggested also for *Gmelinoides fasciatus* (Kalinkina et al., 2022). As a rule, environmentally relevant concentrations of MP are not considered to affect the survival of aquatic organisms upon short exposure (24 h till 7 days) (Bruck and Ford, 2018; Mateos-Cárdenas et al., 2019; Weber et al., 2018). Our results showing slightly increased mortality of amphipods at high LDPE MP concentration allows hypothesize that longer (sub-chronic) exposure times (14 days in our case) and at higher concentrations, MP may affect amphipod survival. However, at lower, environmentally relevant concentrations, this effect may only be seen in certain animal groups. In our case, survival of only *G. lacustris,* which has less mobile lifestyle (see swimming activity data in 3.4) than *G. fasciatus,* was affected.

3.4. LDPE microplastics impact on swimming activity of amphipods

Amphipods may be affected by MP also at sub-lethal level. One evaluated sub-lethal endpoint was amphipod swimming activity. It is noteworthy that the baseline swimming activity of the two amphipod species was significantly different with *G. fasciatus* being much more active (average activity 43.7%) than *G. lacustris* (average activity 12.2%). The greater activity of *G. fasciatus* is likely to increase the ability to escape from areas where water level changes quickly. Indeed, as noted in experimental studies by Kangur et al. (2010), even with major water fluctuations in a lake, the depth *G. fasciatus* could inhabit did not change. This behavior enhances *G. fasciatus* success of relocation, foraging and all in all, its invasive success.

Differences were noted in stress responses LDPE MP induced to the two amphipod species. The more active swimmer *G. fasciatus* was affected by both low and high concentrations of LDPE MP while no significant ($p < 0.05$) effect on swimming of *G. lacustris* was observed (Fig. 4). For both amphipods, the positive control paraquat significantly reduced the swimming activity. Low SiO_2 concentration also induced a significant reduction in *G. lacustris* swimming activity but high SiO_2 exposure had no statistically significant effect on either of the amphipods compared to the negative control group. In case of *G. fasciatus*, SiO_2 treatments (low and high concentration) showed no significant difference from the negative control group (Fig. 4 B).

Our results demonstrated that exposure to LDPE MP decreased the swimming activity of *G. fasciatus* that could signal a potential decrease in the mobility of MP-exposed natural amphipod communities. This in turn could reduce the relocation potential of the animals and cause plastics-affected animals to be eaten in preference. As a result, plastics may enter the food-chain and have an effect on the entire ecosystem.

Only a few studies have demonstrated behavioral effects such as e.g. sensory disruption and affected predation due to MP exposure. In the first case, chemical cues between predator and prey may be impaired (Seuront, 2018), in the latter, predation rates can be decreased (Van Colen et al., 2020) or increased (McCormick et al., 2020) due to MP

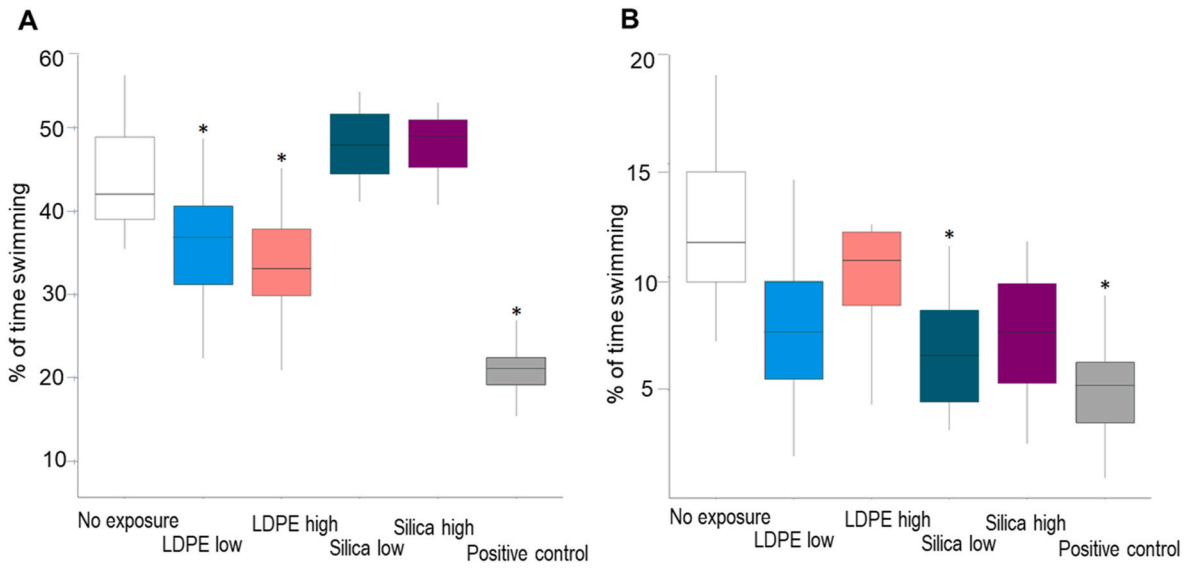

Fig. 4. Swimming activity expressed as time (% of time) spent in motion by (A) *Gmelinoides fasciatus* and (B) *Gammarus lacustris* after 14 days of exposure to low and high concentration of LDPE (2 μg/L and 2 mg/L, respectively), low and high silica (2 μg/L and 2 mg/L of SiO_2), respectively and oxidative stress positive control paraquat. Data for nine replicates are shown using boxplots. * represents a statistically significant difference ($p < 0.05$) in activity between the selected treatment and no exposure conditions.

exposure. For example, in Van Colen et al. (2020) predation rates of benthic filter feeders on bivalve larvae decreased significantly (about 30%) after larvae had been exposed to MP. Authors hypothesized that the lower predation on contaminated larvae can be explained by impaired swimming behavior that drives the larvae away from the inhalant flow field near the filter feeding predator. In McCormick et al. (2020) exposure to MP induced the fish to be bolder, more active, risk-pone, straying further from shelter compared to the control fish thus leading to higher mortality. Authors found that survival increases as fish become more conservative in their behavior (McCormick et al., 2020). The same behavior was also observed for MP-exposed daphnids (Felice et al., 2019). The few behavioral studies carried out with crabs and snails exposed to environmentally relevant concentrations of MP have shown no behavioral response (Cunningham et al., 2021; Doyle et al., 2020). For gammarids, locomotion, altered potentially as a result of oxidative damage and/or altered energy metabolism, has been shown to be a sensitive sublethal toxicity endpoint for nanoparticle exposure (Mehennaoui et al., 2021).

Changes in behavior in some selected links of the food chain (or ecosystem broadly) is nothing new in studies of environmental stressors (e.g., a study in hypoxia demonstrated shifted interspecific reactions in defensive, territorial and predator-prey interactions in Riedel et al., 2014). Such effects can be unpredictable, and currently we lack understanding of potential trophic interactions influenced by MP pollution. Incorporating these data into a stage-structured population model demonstrated that enhanced predation mortality at the larval stage can result in population declines. This indicates that sub-lethal shifts in the behavior of individuals due to human-mediated environmental change can impact species interactions with measurable population-level effects. At a broader level, such changes have the potential to alter higher-order trophic interactions and disrupt aquatic communities (Rearick et al., 2018).

3.5. LDPE microplastics induced oxidative stress

As oxidative stress has been considered one of the most common mechanism of toxicity in environmental responses (Samet and Wages, 2018) and recently, oxidative stress has been shown to be induced also by MP (Han et al., 2022), we used three biomarkers, enzyme superoxide dismutase (SOD), glutathione peroxidase (GPx) and reduced glutathione (GSH), to follow the activities of oxidative stress-related enzymes and GSH depletion in MP-exposed amphipods. Sub-toxic oxidative stress may be monitored by increased enzyme activities and decreased activities of cellular antioxidant GSH.

In *G. fasciatus* significant increase in SOD after exposure to high concentrations of LDPE MP and SiO_2 ($p < 0.001$) was observed (Fig. 5 (A)). Interestingly, the activity of GPx was not significantly affected by LDPE MP in *G. fasciatus* but increased only in response to high SiO_2 exposure and exposure to the oxidative stress control compound paraquat (Fig. 5 (C)). GSH content in *G. fasciatus* was significantly different (reduced; $p < 0.05$) only in low SiO_2 exposure (Fig. 5 (B)). Compared to the oxidative stress biomarkers of *G. fasciatus*, those of *G. lacustris* differed significantly. In *G. lacustris* SOD was significantly increased after treatment with low concentrations of LDPE MP ($p < 0.001$), low and high concentration of SiO_2 ($p < 0.01$ and $p < 0.05$) and paraquat ($p < 0.001$) (Fig. 5 (D)). GPx activity was significantly increased in all the exposures except after exposure to low SiO_2 concentration. GSH was significantly decreased across all the exposures except after exposure to high LDPE MP concentration (Fig. 5 (E, F)).

Overall, our results showed that in the two amphipods, the selected oxidative stress biomarkers responded differently to LDPE MP as well as to the natural particle control SiO_2 (silica) and to the oxidative stress control compound paraquat. For LDPE MP, the most consistent change across the used concentrations and animals was observed for enzyme biomarker SOD. GPx activities were increased only in *G. lacustris*, both at low and high LDPE MP exposures and GSH levels reduced also in case of *G. lacustris* but only at low exposure concentrations. Interestingly, after two week-exposure, paraquat did not induce a significant difference in *G. fasciatus* SOD and GSH. More studies are needed to know whether the dose needs to be adjusted or whether paraquat is not an ideal indicator for this purpose with this species. SiO_2, which was used to account for any general particle effects, induced similar response in SOD measurements but not in GPx and GSH assays. Therefore, it is possible that some of the effects registered for LDPE MP may have been unspecific microparticle effects but others could be attributed to increased load of MP.

The two species had the baseline values of the enzymatic markers evaluated and compared with the control values. There was no significant difference between background and control values in the biomarkers of either species ($p < 0.05$). Furthermore, the baseline values of the two species were statistically similar, with the exception of SOD

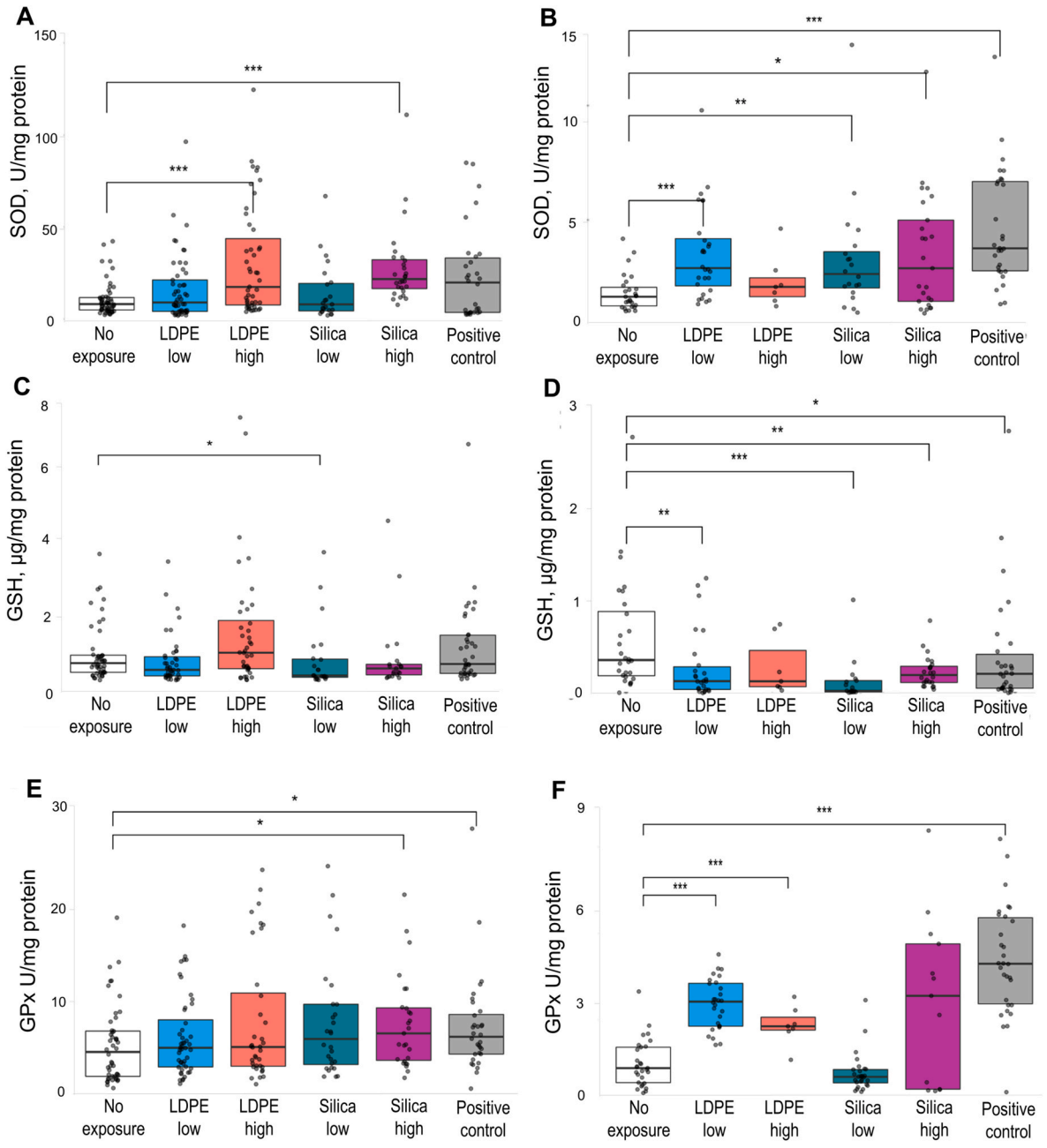

Fig. 5. Changes in oxidative stress markers in response to 14 days of low (2 µg/L) and high (2 mg/L) polyethylene microplastics (LDPE MP) exposure, low or high natural silica (2 µg/L or 2 mg/L of SiO_2, respectively), and oxidative stress positive control paraquat at 1 µg/L. The activity of SOD in *Gmelinoides fasciatus* (A) and *Gammarus lacustris* (B), the level of GSH in *G. fasciatus* (C) and *G. lacustris* (D) and the level of GPx in *G. fasciatus* (E) and *G. lacustris* (F) are expressed as units (U) or µg per mg protein. Data of 15 replicates are shown. * represents a statistically significant difference ($p < 0.05$) in oxidative stress markers between the selected treatment and no exposure conditions, ** represents $p < 0.01$, *** represents $p < 0.001$.

activity, which is naturally higher in *G. fasciatus*. SOD is an important antioxidant cellular defense and due to the higher activity in *G. fasciatus* we can conclude that this may be the reason why this species was less affected by LDPE MP exposure compared to *G. lacustris*.

Due to the lack of data, comparing the results with other studies is challenging. However, studies with other aquatic invertebrates show neither significant induction of catalase (CAT) nor glutathione-s-transferase (GST) activity in MP-exposed mussels (Avio et al., 2015), snails and crustaceans (Trestrail et al., 2020). Our results showed that in general, LDPE MP exposures induced a higher level of oxidative stress response in *G. lacustris* than in *G. fasciatus*. Since the two species ingested-egested LDPE MP at comparable amounts and no cellular uptake of MP has been reported for gammarids (Blarer and Burkhardt-Holm, 2016), it cannot be outruled that the source of oxidative stress, especially for *G. lacustris* that also had higher mortality rate, was not MP-induced. Whether the recorded oxidative stress translates into higher vulnerability of *G. lacustris* towards LDPE MP exposures remains to be further determined by evaluation of potential oxidative damage (e.g. lipid peroxidation) that has been shown in literature (Chen et al., 2022). In general, lower resistance of *G. lacustris* to threats is demonstrated by the competitive dynamics observed between the two amphipod species, where usually *G. lacustris* ends up at a disadvantage (Berezina, 2009).

4. Conclusion

The main goal of this study was to analyze and compare the effects of low density polyethylene microplastics (LDPE MP) on two amphipods, *Gmelinoides fasciatus* and *Gammarus lacustris* that in a natural system compete for their habitats. In natural conditions, the complete dominance of the former has been shown to occur within about 10 years. Mortality, swimming activity and oxidative stress biomarker response in amphipods after sub-chronic exposure (14 days) to both, environmentally relevant concentrations and to significantly higher concentrations of LDPE MP was analyzed. We were able to confirm that environmentally relevant LDPE MP concentrations triggered small but significant effects in all the analyzed endpoints: mortality and behavior (swimming) in at least one of the tested animals. Regarding the enzymatic biomarkers, GPx showed changes that may be indicative of exposure to MPs, but potential oxidative damage needs further research. Importantly, we found that the two species differed in their response to LDPE MP exposure: *G. fasciatus* changed its swimming activity and *G. lacustris* showed increased level of oxidative stress. Therefore, in MP exposure conditions, *G. fasciatus* may lose its normally high relocation potential and plastics-affected animals may be eaten in preference. On the other hand, higher oxidative stress response in *G. lacustris* may increase its inherent vulnerability to invasive species and thus, further reduce its natural competitiveness. These differences in the responses of the two amphipod species to LDPE MP require more detailed studies in the future.

First studies are appearing that indicate potential ecosystem-level threat of MP pollution at environmentally relevant concentrations. Therefore, we advise policy makers to advance from a careful and cautious (slow) approach that handles MP pollution as an emerging contaminant to a specific action plan and measures. Potentially vast effects of MP need to be addressed in close future in policy planning.

Credit author statement

Bárbara Rani-Borges: Conceptualization, Methodology, Formal analysis, Data curation, Investigation, Visualization, Writing – original draft, Writing – review and editing, Funding acquisition. **Richard Meitern:** Methodology, Formal analysis, Data curation, Writing – review and editing, Supervision. **Paul Teesalu:** Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review and editing. **Merilin Raudna-Kristoffersen:** Conceptualization, Writing – review and editing. **Randel Kreitsberg:** Conceptualization, Methodology, Data curation, Writing – review and editing, Supervision. **Margit Heinlaan:** Conceptualization, Methodology, Data curation, Writing – review and editing, Supervision, Funding acquisition. **Arvo Tuvikene:** Methodology, Supervision, Funding acquisition. **Angela Ivask:** Conceptualization, Methodology, Data curation, Writing – review and editing, Supervision, Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.136599.

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