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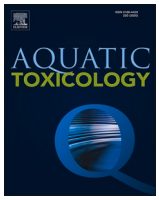

Exposure of the amphipod *Hyalella azteca* to microplastics. A study on subtoxic responses and particle biofragmentation

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ABSTRACT

Microplastics are widespread pollutants in the environment and are considered a global pollution problem. Microplastics mostly originate from larger plastics and due to environmental conditions are undergoing constant fragmentation processes. It is important to understand the fragmentation pathways, since they play a key role in the fate of the particles, and also directly influence toxicity. Amphipods are potential inducers of plastic debris fragmentation. Here, *Hyalella azteca* was exposed to different concentrations (540, 2700, 5400 items/L) of 24.5 μm polystyrene microplastics (PS-MP) for 7 days. After exposure, oxidative stress, particle size reduction, and mortality were checked. No significant mortality was seen in any of the treatments, although changes were recorded in all enzymatic biomarkers analyzed. It was observed that throughout the ingestion and egestion of PS-MP by *H. azteca*, particles underwent intense fragmentation, presenting a final size up to 25.3% smaller than the initial size. The fragmentation over time (24, 72, 120, 168 h) was verified and the results showed a constant reduction in average particle size indicating that *H. azteca* are able to induce PS-MP fragmentation. This process may facilitate bioaccumulation and trophic particle transfer.

1. Introduction

Plastic is a general name for the composite of numerous materials and products that are indispensable to society. Due to lack of proper management and its intense use worldwide driven by the widespread consumerism system throughout today's society, plastic has become a pollution problem of global proportions (Geyer et al., 2017; Horton and Dixon, 2017; Sharma et al., 2020). After their disposal and through various routes, plastics eventually reach water bodies, where they are exposed to environmental conditions, biotic and abiotic, capable of inducing the degradation and fragmentation of plastics into smaller pieces (Sorasan et al., 2022). Temperature, pH, exposure to ultraviolet rays, interactions with animals, as well as friction with rocks, sediment, wind, and current, are the main factors that can lead larger plastics to become so-called microplastics (secondary), with a size of up to 5 mm (Klein et al., 2017; Koltzenburg et al., 2017; Worm et al., 2017).

Microplastics are pollutants that are now widespread in all aquatic ecosystems, with their occurrence reported in marine, estuarine, freshwater environments, in polar regions, in sewage treatment plants, and in drinking water (Cincinelli et al., 2017; Luo et al., 2019; Murphy et al., 2016). The ecotoxicity of microplastics is influenced by numerous factors, making it challenging to determine which ones are more or less harmful to different organisms and ecosystems. Ecotoxicological tests conducted under controlled conditions can help to assess the expected outcomes based on the microplastics' characteristics. Ecotoxicological tests with microplastics have been conducted on a wide range of aquatic organisms such as fish (Azizi et al., 2021), crustaceans (Rani-Borges et al., 2022), mollusks (Ding et al., 2022), and mussels (Joyce and Falkenberg et al., 2023). These tests aim to assess the potential effects of microplastics on different aspects of the organisms' biology, including

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growth, reproduction, survival, and sublethal endpoints (Doyle et al., 2022). The tests are conducted under controlled laboratory conditions to minimize other confounding factors that could affect the results, so that it is possible to attribute the outcomes to a specific factor.

In recent years, studies have focused on understanding the transport and fate of microplastics through water bodies (Li et al., 2018; Krause et al., 2021), as they are determining factors in the distribution and concentration of particles in the medium (Eerkes-Medrano et al., 2015). In addition, these processes also directly influence the ways in which this pollutant interacts with biota. However, a factor that has not yet received due attention refers to the trends of particle size reduction and fragmentation, being essential to predict the mechanisms involved, considering that it is a factor that will change the dynamics of microplastics in environmental matrices in addition to its ecotoxicity potential. According to critical analysis by Uzun et al. (2022), for building mathematical models to predict microplastic transport, smaller microplastics show higher velocities in aqueous media, suggesting that the smaller the particles, the greater the distances reached. Besides that, the toxic potential of microplastics can be increased as the particles are also smaller, this occurs since smaller particles are able to interact with a larger number of organisms (He et al., 2022; Sharma et al., 2022).

Benthic macroinvertebrates, play a key role in the ecosystem as intermediaries between primary producers and higher trophic levels (Tudor et al., 2016). Studies on microplastics with amphipods are still largely limited to endpoints of ingestion, egestion and mortality (Au et al., 2015; Blarer and Burkhardt-Holm 2016; Gerhardt, 2020; Iannilli et al., 2019; Yardy and Callaghan, 2020) superficially highlighting the effects on the benthic organisms. Further investigations on subtoxic responses following exposure to microplastics are required. Changes in bioindicators associated with oxidative stress can help to understand the impact of microplastics on benthic species (Jeyavani et al., 2022a; Rani-Borges et al., 2022). In this study the freshwater amphipod Hyalella azteca was exposed to different concentrations (540, 2700, 5400 items/L) of 24.5 μm polystyrene microplastics for 7 days to elucidate the effect on different oxidative stress biomarkers such as CAT, SOD, GST, and MDA. The study of impacts and exposure to microplastics by benthic species, such as the amphipod *Hyalella azteca*, may help to understand the consequences of this interaction, especially with regard to changes in bioindicators associated with oxidative stress and in the ability to fragment plastic particles. Thus, the results of exposure to microplastics by species such as the amphipod *H. azteca*, can be a good indicator of environmental quality and help understand the consequences of this interaction for the ecosystem as a whole.

Considering previous studies that recorded microplastic fragmentation induced by amphipod species *Gammarus duebeni* (Mateos-Cárdenas et al., 2020) and the similar nature of *H. azteca*, we hypothesized that *H. azteca* may exhibit similar behavior in the face of MP exposure. Therefore, the fragmentation of the PS-PMs by *H. azteca* was investigated during the 7 days exposure studies. Also, due to potential physical and chemical interactions between the MP and the organism's physiological processes, it is hypothesized that exposure to microplastics will result in subtoxic responses in *Hyalella azteca*. This hypothesis is supported by the fact that microplastics have been found to accumulate in the digestive tract of aquatic organisms (Keerthika et al., 2023; Zavala-Alarcón et al., 2023), which can interfere with nutrient absorption and oxygen exchange, and also by the potential release of harmful additives and breakdown products from the plastic particles.

2. Material and methods

2.1. Microplastic

According to global industry data, polystyrene (PS) is one of the most produced types of plastic in the world, accounting for approximately 10% of all non-fibrous plastic (Geyer et al., 2017). The microplastic tested purchased from Sigma-Aldrich, was crystal spherical primary PS, with a particle size of 24.5 ± 3.9 μm (standard deviation, SD). The particle size was confirmed from measuring 60 particles (in triplicate) on a Zeiss Discovery V12 steromicroscope at 100x magnification. For the exposure test, a suspension of the microplastics in ultrapure water was made to reach concentrations of 540, 2700 and 5400 items/L. The number of particles per concentration was confirmed by counting the lowest concentration tested in triplicate (540 ± 6.0). The reason for selecting the lowest concentration is that at low concentrations, the particles are better dispersed and less likely to aggregate, which can affect the accuracy of particle counting. Additionally, counting the particles at the lowest concentration provides a baseline measurement for the concentration of particles. This baseline measurement can be used to compare and validate the concentrations of particles in the higher concentrations tested.

The chemical characterization of the polymer was confirmed using a micro-Fourier transform infrared spectroscopy (micro-FTIR). The FTIR spectra were measured in a Bruker spectrometer, Alpha model, in region of 400–4000 cm^{-1}, with standard KBr beamsplitter and high sensitivity DLATGS detector. The spectra were recorded with the ATR (Attenuated Total Reflection) module: ATR Platinum, equipped with a diamond crystal as a reflective element. The spectra were obtained with 128 accumulations and with a resolution of 2 cm^{-1}.

2.2. Test organism

Amphipods are considered good models to assess the potential toxicity of contaminants and pollutants due to the role they played in the trophic chain as an intermediary between primary producers and higher-level consumers (Glazier, 2014; Melo and Nipper, 2007). The amphipod species chosen for the tests was the benthic crustacean *Hyalella azteca* Saussure, 1858. These species are abundantly distributed in aquatic ecosystems and cultures are easy to maintain in the laboratory (Borgmann et al., 2005; Péry et al., 2005). In addition, amphipods are effective in ingesting particulate matter, including anthropogenic polymers (Driscoll et al., 2021; Yardy and Callaghan, 2020).

Adult organisms of *H. azteca* used in the present study were from the Institute of Biosciences (IB) at University of São Paulo. *H. azteca* cultures were maintained in 3 L glass containers. Containers were filled with 2.5 L of MS medium rich in mineral salts (Murashige and Skoog, 1962) and the cultured proceedings were based on the technical standards for toxicity tests with *Hyalella* spp from the Brazilian Association of Technical Standards NBR 15.470 (ABNT, 2007).

2.3. Preparation of experimental diets

A suspension of flaked fish feed (TetraMin) was prepared with ultrapure water to a concentration of 5 g/L, then 100 μL of the fish feed suspension was mixed with the microplastic suspension at the three concentrations tested (540, 2700, 5400 items/L). As surface waters can contain microplastics in quantities as high as grams per liter, such as the recorded amount of 2.6 g/L with particle sizes less than 2 mm (Kowalczyk et al., 2017), all concentrations tested in our study can be considered environmentally realistic. Concentrations similar to those used in the present study have already been registered in freshwater environments in water and sediment samples in Australia (Kowalczyk et al., 2017; He et al., 2020), Germany (Klein et al., 2015), Canada (Vermaire et al., 2017) and China (Ding et al., 2019).

The food mixture with PS-MP was prepared in porcelain containers and dried in a 60 °C oven for 24 h to form a pellet. This pellet formed inside a porcelain container was placed on the bottom of each test flask. Our approach was chosen in order to ensure that the PS particles remained at the bottom of the flasks and did not rise to the surface, thus achieving optimal experimental conditions.

2.4. Exposure of Hyalella azteca to PS microplastics

Prior to the exposure period, 5 individuals of *H. azteca* were distributed in the test flasks for a 48-hour acclimation period without access to food. After this period, it was verified if there were any individuals damaged as a result of handling. Organisms standing still for more than 10 s were excluded from the test and replaced by organisms acclimatized and in ideal conditions of locomotion. The test flasks were glass-made with 600 mL capacity and contained 250 mL of MS medium (Murashige and Skoog, 1962). Twelve flasks were placed under controlled conditions at 24 ± 1 °C, pH 7.0–7.6, total hardness 40–48 mg, conductivity 190–250, dissolved oxygen > 5.0 mg/L, and 16:8 (light: dark) natural lighting photoperiod without aeration for 2 days of acclimation followed by 7 days of exposure. To avoid contamination, all flasks were kept covered with metal lids during the experiment.

After acclimation, the organisms were exposed to microplastics via food for a period of 7 days. The porcelain containers with the food and microplastics (except for the negative control container that consisted of food only) were positioned at the bottom of the flasks. The three concentrations and the negative control were tested in triplicate containing 5 *H. azteca* individuals in each. After the exposure period, the reduction in diameter of the PS spheres was verified. This experiment corresponds to the first experiment.

To ensure consistency of the results and to construct a curve with the fragmentation over time, the experiment was repeated for the lowest concentration (540 items/L). The experiment (corresponding to second experiment) was composed of treatments in triplicate with 5 organisms in each. Each treatment (in triplicate) was finished according to the periods to be analyzed: 24, 72, 120, and 168 h. After the exposure periods, the reduction in diameter of the PS spheres was verified.

At the end of 7 days, the remaining organisms were removed and washed with distilled water to proceed with the following analyses. For the analysis of oxidative stress biomarkers, 4 animals were used from each replica (first experiment). Additionally, organisms from the second experiment ($n = 3$ each replicate) were selected to be observed under an inverted light microscope (Leica DMi8) with fluorescence to determine whether they had ingested any particles.

During the tests, the neonates were removed from the test flasks to avoid alteration in the results due to population increase. The neonates were gently removed using a Pasteur pipette, with great attention paid to minimizing the amount of medium removed along with them.

2.5. Oxidative stress biomarkers

After 7 days of exposure, 3 organisms (1 of each replica) were used for each homogenate preparation. Two homogenates were prepared, and analyzed in triplicate. The organisms were then transferred to 2 mL microtubes containing 600 μL of 100 mM potassium phosphate buffer pH 7.4 and euthanized in a cold bath for 1 h. Using a glass rod, the organisms were macerated and then centrifuged at 4000 g for 30 min at 4 °C and then stored under refrigeration (−80 °C). Protein concentration was determined according to the Bradford assay (1976).

2.5.1. Superoxide dismutase (SOD)

SOD activity was analyzed by the reaction of pyrogallic acid with the triplicated samples, observed at 420 nm (Marklund and Marklund, 1974). In 2 mL microtubes, 1.3 mL of tris-EDTA buffer (5 mM, pH 8.0), 60 μL of the homogenate, and 75 μL of the pyrogallol solution (15 mM) were added and then homogenized vigorously for 20 s. The assays were incubated for 30 min in the dark at 25 °C. After incubation the oxidation reaction was stopped by adding 65 μL of 1 N HCl. The same preparation was performed for the blank, and 60 μL of 100 mM potassium phosphate buffer pH 6.5 was used. The SOD activity was determined by the ability to inhibit the reduction of pyrogallol by superoxide radicals by 50% expressed in U/SOD.

2.5.2. Catalase (CAT)

CAT activity was evaluated using the enzyme assay described by Prado et al. (2021). Assays were performed in triplicate using 100 mM potassium phosphate buffer (7.0) and 20.0 mM hydrogen peroxide (H_2O_2) in a 1:1 (v/v) ratio and 20.0 μL of the homogenate. The activity was monitored by the consumption of H_2O_2 resulting in the decline in absorbance at 240 nm for 3 min. One unit of CAT activity was defined as the consumption of 1 nmol of H_2O_2 /min.

2.5.3. Glutathione S-transferases (GST)

GST activity was determined as described by Prado et al. (2021) with modifications. Assays were performed in triplicate by adding 490 μL of 100 mM potassium phosphate buffer (pH 6.5), 490 μL of the mix solution (9.5 mM reduced glutathione (GSH) /1-chloro-2,4-Dinitrobenzene (CDNB) 1.0 mM, diluted in 100 mM potassium phosphate buffer (pH 6.5) and methanol, respectively) and 20 μL of homogenate. The same solution was prepared for the blank using buffer. GST activity was monitored through the formation of S-(2,4-dinitrophenyl) conjugated glutathione, and expressed as μmol/CDNB-GSH/min mg protein, by increasing absorbance at 340 nm for 5 min.

2.5.4. Malonaldehyde (MDA)

Lipid peroxidation damage was assessed using MDA levels as described by Campos et al. (2014), with adaptations. Assays were performed using 0.4% thiobarbituric acid (TBA) diluted in 100 mM potassium phosphate buffer (pH 2.5). In a 10 mL test tube, 2 organisms (*H. azteca*) from each replica were added and macerated with 500 μL of mM potassium phosphate buffer (pH 7.4). Subsequently 1 mL of 0.4% TBA was added, homogenized, and incubated in a water bath at 95 ± 1 °C for 45 min. After being cooled in an ice bath, the samples were centrifuged at 3000 rpm for 5 min at 25 °C and read by wavelength 532 nm. The blank solution was prepared from 500 μL of 100 mm potassium phosphate buffer pH 7.4 and 1 mL of 0.4% TBA. The same process was performed for the standard control, and 500 μL of 4.5 mM 1,1,3,3-tetraethoxypropane (TEP) and 1 mL of 0.4% TBA were added. The results were expressed in nmol/mL of MDA.

2.6. Biofragmentation assays

Two biofragmentation assays were performed. The first experiment comprised on the initial and final size analysis (after 7 d). In contrast, the second experiment examined the mean size reduction of the microplastics over time (up to 7 d).

After exposure period KOH was added directly to the MS medium until it reached the concentration of 10%. Digestion of the organic matter was done in an oven at 60 °C for 48 h. Then the medium was completely filtered through a vacuum system onto 47 mm Whatman glass fiber filters (GF/C) with a pore size of 0.7 μm. The filters were investigated under a Zeiss Discovery V12 stereomicroscope at 100x magnification to measure particles ($n = 20$ per replicate) and to check if there was a reduction in mean size.

After diameter measurement, the glass fiber filters were cut with a straight razor blade. The samples were coated with gold until it forms a layer 5 nm thick using a vacuum sputter coater before being placed into the chamber. The gold coating process ensures a uniform coating on the sample surface, which enhances the quality of the images obtained during analysis. The morphology of these microplastics was characterized by scanning electron microscopy (FESEM model JEOL JSM-7401F).

For the second experiment, 12 flasks were prepared with 5 amphipods each. After 24, 72, 120 and 168 h three flasks were randomly selected and the reduction in diameter of the PS spheres was verified as described before. A particle control treatment was also performed to ensure that the PS-MP size did not undergo any reduction due to the period it remained in the culture medium and the organic matter digestion process. As with the other treatments, particle control was performed in triplicate during the 7-day period under concentration of

540 items/L, however without the presence of organisms. At the end of day 7, the medium went through the process of digestion of organic matter as applied to the other treatments.

2.7. Mortality

During exposure, the flasks were monitored for amphipod mortality at 48, 96, and 168 h. Mortality was expressed as a percentage of the initial number of organisms in the experiment.

2.8. Statistical analysis

To determine whether there were significant effects between exposure time, MP concentration, and the endpoints analyzed (PS particle size reduction, the mortality and oxidative stress endpoints), Dunnett's statistical test was employed in addition to descriptive analyses. Fisher's exact test was used to compare whether there was a difference between the results of the treatment groups according to concentration. All analyses were performed in triplicate and statistical significance was accepted at the $p < 0.05$ level.

3. Results and discussion

3.1. Characterization of PS-MP

MP used in the present study were green fluorescent PS microbeads of 24.5 μm diameter and particle density of 1.02–1.05 g/cm^3. The microbeads (excitation wavelength of 480 nm) were verified by a micro-FTIR spectrometer (Fig. 1). The regular morphology and expected size of the particles were confirmed by observing and measuring 60 particles (in triplicate) under a stereomicroscope. The measurements showed that the mean diameter was 24.5 ± 3.9 μm.

3.2. Oxidative stress biomarkers

The study of enzyme biomarkers has been shown to be an important endpoint for investigating subtoxic impacts on biota caused by various pollutants, including microplastics (Han et al., 2022; Samet and Wages, 2018). Despite this, there are few studies investigating the effects of microplastic exposure with respect to biochemical changes. Studies with an approach focused on biochemical and molecular parameters were not found in the literature for the species in evidence here, indicating a worrying gap in knowledge. In this work we investigated whether there were changes in the levels of SOD, CAT, GST, and MDA after 7 days of exposure to PS-MP, under the concentrations of 540, 2700, 5400 items/L.

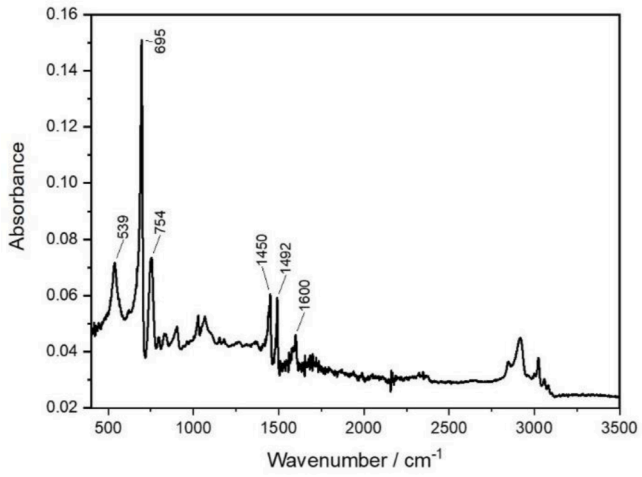

Fig. 1. Fourier transform spectroscopy (FTIR) confirming the type of polymer as polystyrene (PS).

The activities of SOD, CAT, and GST, and the levels of MDA and in *H. azteca* treated with PS are shown in Fig. 2(A-D). Significant changes ($p < 0.05$) were observed in all enzyme markers, varying according to the concentrations tested. SOD and GST showed difference compared to the negative control only at the concentration of 2700 items/L with 21 and 72% increase, respectively. MDA and CAT analyses showed altered levels with significant differences in all tested concentrations with increased enzymatic activity of 136, 159 and 142% in MDA and 57, 60 and 44% in CAT at concentrations of 540, 2700, 5400 items/L, respectively when compared to the control.

Contrary to what might be expected, the lowest concentration (540 items/L) and the highest concentration (5400 items/L) induced similar biochemical responses. Surprisingly, the intermediate concentration was able to induce changes in all enzymatic biomarkers tested, while the same effect was not visualized in the treatment with the highest concentration. Further studies are needed to understand the mechanisms involved in this process. Little is known about the effects at the biochemical level in *H. azteca*, however, we attribute the results obtained in the present study to the possible capacity of autoregulation of the species, which is usually induced in stress situations and which can be more pronounced according to the concentration of the pollutant present in the environment.

PS-MP-induced oxidative stress in other species has already been reported in algae (Hazeem et al., 2020; Zheng et al., 2021), water column crustaceans (Lin et al., 2019), mussels (Avio et al., 2015), and fish (Lee et al., 2019; Solomando et al., 2020), for example. Yet, studies investigating the effects of microplastics on CAT and GST levels showed that there were no significant changes in these bioindicators (Avio et al., 2015; Trestrail et al., 2020). In other studies, it was also found that the type and size of the analyzed polymers directly influenced the biochemical responsiveness of organisms (Espinosa et al., 2019; Kang et al., 2021; Yang et al., 2020).

In the present study, SOD activity showed the least modifications compared to the control group, suggesting efficient elimination of superoxide radicals (O_2^-) through their conversion into H_2O_2 (Sies, 2017). This result is corroborated by the increased CAT activity, observed in Fig. 2(B-D). However, the increased levels of all biomarkers indicate that the dose-response relationship can occur up to a certain limit, as observed in Fig. 2, going on to inhibit and/or demand greater redox activity, a process known as adaptation mechanisms (Ighodaro and Akinloye, 2018). Studies have shown that high concentrations of PS-MP can promote redox process inhibition by reducing the enzymatic activity of GST, SOD, and CAT (Hamed et al., 2020; Kim et al., 2021), as observed in the present study. Usually, the increase of GST is associated with metabolization and elimination process of toxic compounds (step II), playing a great role as a main biomarker of environmental quality (Prado et al., 2021). Probably the decrease in GST activity (Fig. 2B) is associated with increased responses of the enzymes SOD and CAT, which act as the first line of antioxidant defense against reactive oxygen species (ROS) produced (Ighodaro and Akinloye, 2018). Similarly, it is also observed that MDA levels showed a greater increase, as the dose-response, suggesting greater oxidative damage to tissues (Hamed et al., 2020).

Here, we use only environmentally realistic concentrations, aiming to simulate a current scenario of microplastic pollution and get answers about what is possibly happening at the present time to the benthic biota. However, higher concentrations of microplastics may cause physical interference (e.g., attachment onto organism surface or compete with food) and induce greater levels of toxic effects, including molecular modifications at long-term exposures (Malafaia et al., 2020; Moreschi et al., 2020). These events could theoretically lead to increased oxidative stress, which were not observed in all conditions tested here.

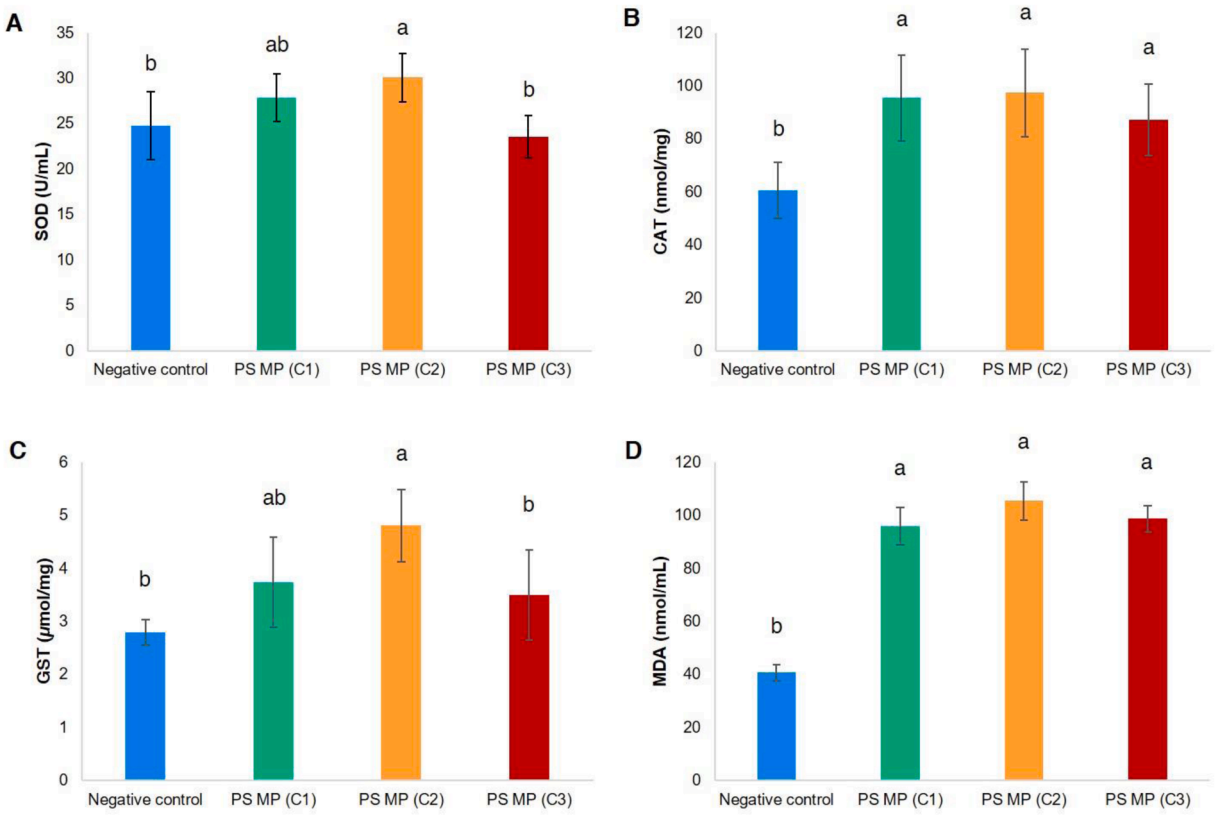

Fig. 2. Changes in oxidative stress markers in response to 7 days of polystyrene microplastics (PS-MP) exposure at three different concentrations (C1: 540, C2: 2700, C3: 5400 items/L). The levels of (A) SOD, (B) CAT, (C) GST and (D) MDA in *H. azteca* are shown accordingly to protein content. Equal letters indicate no significant difference and different letters indicate a significant difference ($p < 0.05$) between treatments (ANOVA and Tukey's Post Hoc Test).

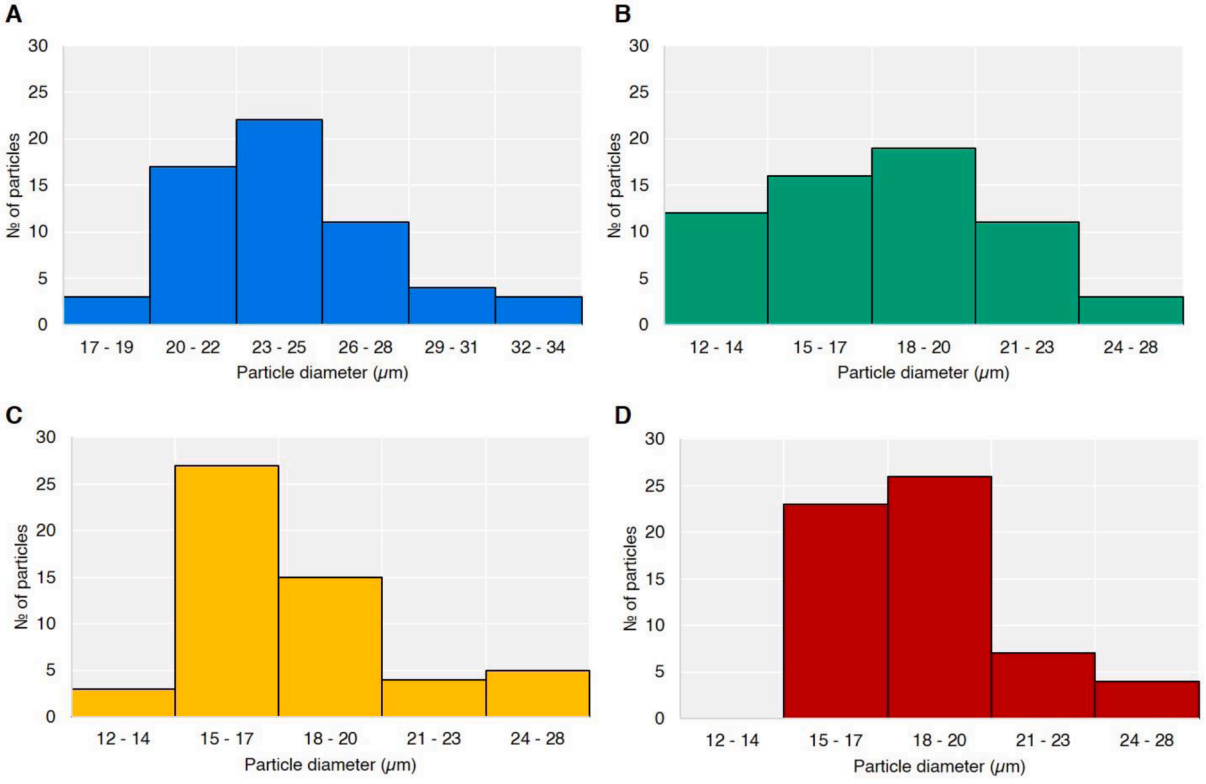

Fig. 3. The particle size histogram obtained from measurement of polystyrene microplastics (PS-MP; $n = 60$) before (A) and after 7 days of exposure at three different concentrations. C1: 540 (B), C2: 2700 (C), C3: 5400 items/L (D).

3.3. Biofragmentation of PS microplastics by Hyalella azteca

The biofragmentation of PS-MP by *H. azteca* was checked after exposure period of 168 h (7 days) under different concentrations (540, 2700, 5400 items/L). The animals were removed and the medium was analyzed in its entirety.

The initial hypothesis, that particles not ingested by the organisms would not undergo any change during the exposure period and through the organic matter digestion step, was confirmed by performing the particle control treatment which showed that there was no significant reduction in the size of the spheres (which showed a final size of 22.31 μm ± 2.85 at the concentration of 540 items/L). Thus, any reduction in average particle size can be considered as coming from the interaction with the amphipods.

Comparison of the sizes of PS-MP ingested and after egestion (recovered from MS medium), showed that the sizes of these were smaller than the initial size (see particle size histogram in Fig. 3). After 7 days of exposure, the mean particle diameters were 18.35 μm ± 3.22, 18.58 μm ± 3.07 and 19.29 μm ± 2.66 at concentrations of 540, 2700, 5400 items/L, respectively (Fig. 4A). Dunnett's test showed that all treatments showed final size statistically different from the control ($p < 0.05$), while Fisher's test showed that there was no difference between the concentrations tested. The average decrease in particle diameter over time was obtained by observing the particle size of the lowest concentration (540 items/L) at 4 time points, namely 24, 72, 120, and 168 h. The graphical representation was made with the percentage size reduction compared to the initial average size (Fig. 4B).

Considering the observed difference in diameter according to time, this difference may indicate that the concentration is a factor that modulates the fragmentation process. It is assumed that these differences occurred because the organisms ingested the same particles more than once, and at higher concentrations this may have happened less often. As shown by these results (Fig. 4), the lower the concentration and the longer the exposure time, the greater the fragmentation of the microplastics. Furthermore, in view of the fragmentation curve, it is possible to suggest that studies with a longer exposure period may result in even higher fragmentation rates. In this sense, it is important that further studies are conducted within the scenario proposed here.

When analyzing the samples with scanning electron microscopy, it was possible to verify that the PS spheres suffered modification in their physical structure after the exposure period (Fig. 5). The particles showed visible signs of physical changes, such as appearance of cracks, fissures, and irregular edges. These changes in the microplastics' morphology may indicate that the organisms are interacting with the particles and causing damage to their structure. One of the primary mechanisms responsible for the surface morphological changes of microplastics is the fragmentation of the particles due to mechanical stress, which can cause cracks, fissures, and irregular shapes on their surfaces (Guo and Wang, 2019). Another process that can result in surface changes is depolymerization, which occurs when extracellular enzymes break down the material through biodegradation (Cholewinski et al., 2022). These surface morphological changes can have significant implications for the toxicity, bioavailability, and transport of microplastics in aquatic environments (Hüffer et al., 2018; Kowalski et al., 2016).

Although some studies suggest that ingestion rate is dose-dependent (Weber et al., 2018), our results suggest that there is a limit to particle ingestion by *H. azteca*, likely as a result of body size and the limitations of the length of the digestive tract. Thus, the limit on ingestion rates is what resulted in the least reduction in average particle size at the end of the exposure period, especially the higher the concentration employed.

In order to confirm whether ingestion of the spheres occurred, individuals of *H. azteca* were observed under inverted microscope with fluorescence (Leica DMi8) (Fig. 6), and it was possible to observe internally lodged particles. Ingestion and egestion of particles by benthic organisms under natural and controlled conditions have been reported previously, including for microplastics with the same size range used in the present study (Prata et al., 2023; Queiroz et al., 2022; Sfrisoet al., 2020). The biofragmentation rate, however, is a missing piece of data. The first studies addressing this mechanism, investigated the ability of polyethylene microplastics to fragment by Antarctic krill (*Euphausia superba*) (Dawson et al., 2018) and *Gammarus duebeni* (Mateos-Cárdenas et al., 2020), also a freshwater amphipod, confirming such a hypothesis. Thus, the fragmentation of plastics and microplastics induced by aquatic organisms can be given as pertinent, at least for freshwater amphipods, and the development of mathematical models that can assist in obtaining data to help predict trends in particle size reduction is needed.

As bottom-feeding invertebrates, *H. azteca* feed on organic matter present in the sediments. These organisms feed in a continuously mood, having the ability to convert organic matter into fecal pellets in a short time (Hargrave, 1970). It has been shown by longstanding studies that *H. azteca* can also ingest sediment particles (Hargrave, 1970), which may be an indication that these organisms are not selective in their diet, and may also ingest solid pollutants present in the environment, in fact, as demonstrated in the present work, where *H. azteca* ingested PS-MP. Khan et al. (2019) reported similar findings in *H. azteca* individuals exposed to tire wear particles, with ingestion occurring within the first hour of exposure. The authors further observed that the particles remained in the intestine for 24–48 hours, and that the gastrointestinal tract was clear of particles after a depuration period of >48 h in a clean

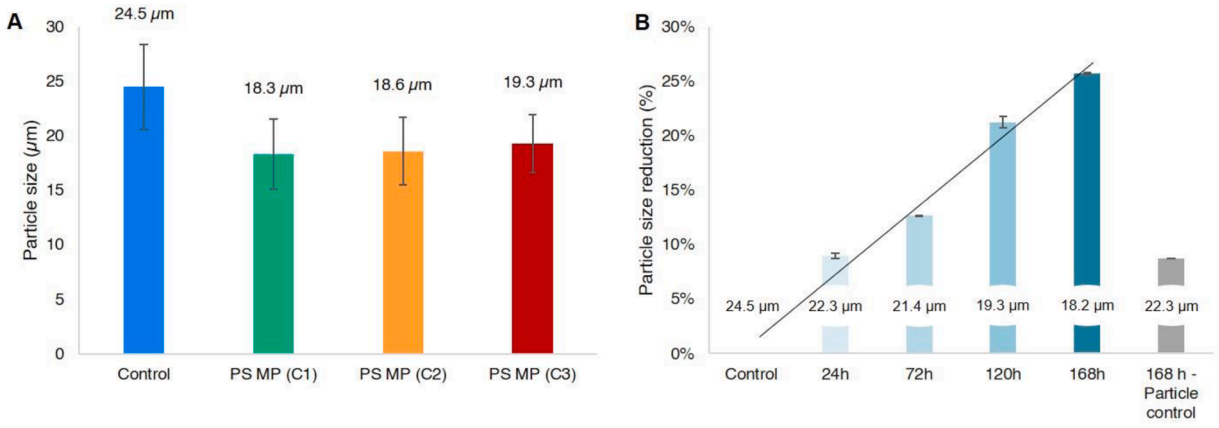

Fig. 4. (A) First experiment: fragmentation of polystyrene microplastics (PS-MP; $n = 3$) after 7 days of exposure at three different concentrations (C1: 540, C2: 2700, C3: 5400 items/L). Data are shown as mean for $n = 20$ measurements ± SD in $n = 3$ replicates. (B) Second experiment: the average decrease in particle diameter (%) of polystyrene microplastics (PS-MP) over time (24, 72, 120 and 168 h) compared to the initial mean size (24.5 μm) observed after interaction with Hyalella azteca organisms under the exposure concentration of 540 items/L.

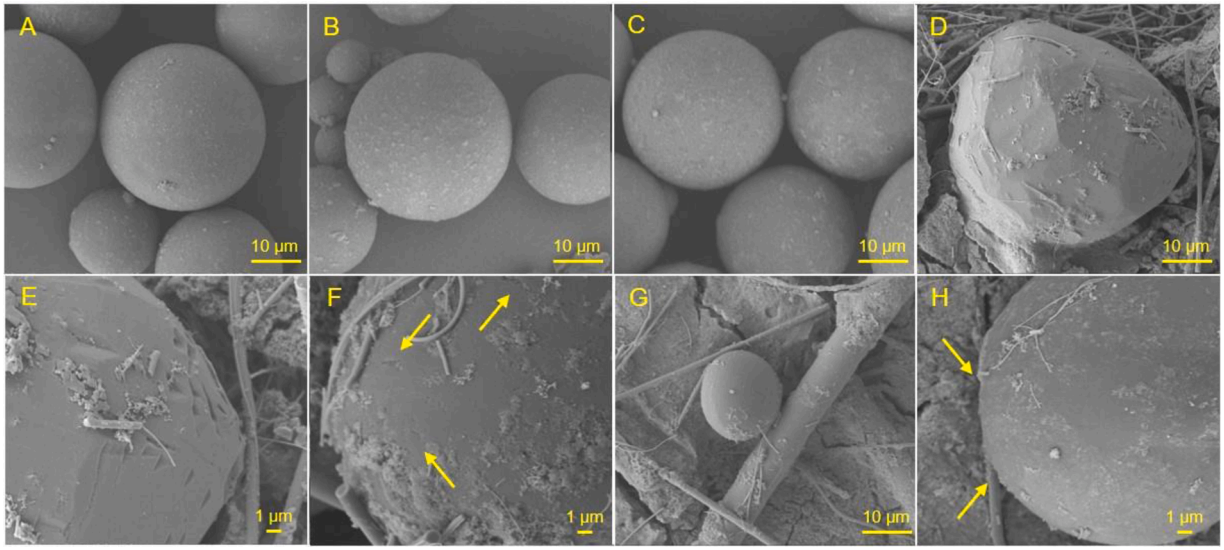

Fig. 5. Scanning electron microscope of polystyrene microplastics after exposure to Hyalella azteca under concentration of 340 items/L. (A) Original particles; (B) particles after dried at 60 °C for 24 h prior to application; (C) MPs from the particle control treatment; (D-H) particles recovered from the medium after 7 days exposure with Hyalella azteca, showing irregular edges and cracks. Arrows highlight points of interest, indicating cracks, fissures, and rough edges. Scale bars represent 10 (A-D, G) or 1 µm (E, F, and H).

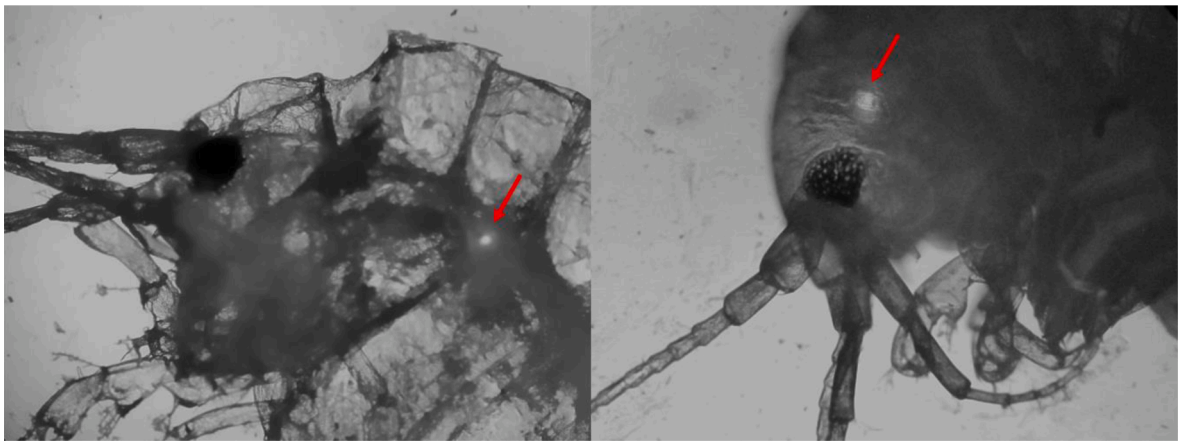

Fig. 6. Ingested fluorescent polystyrene microplastics (PS-MP) inside Hyalella azteca, captured using an inverted light microscope with fluorescence after 7 days of exposure to a lower concentration (540 items/L) of the second experiment.

medium. These results suggest that the ingestion of particles by these organisms may serve as a significant pathway for introducing pollutants into the aquatic food chain, as there is no depuration period in the environment, since the influx of pollutants is constant, despite being heterogeneous.

H. azteca have a digestive tract composed with a set of food-crushing plates and ossicles, the gastric mill, while the pyloric cuticle forms a complex straining and pressing mechanism (Schmitz and Scherrey, 1983). After passing through the grinding system, the particulates pass through the hepatopancreas, the structure where digestive enzymes are secreted, in addition to the enzymatic reaction, this organ has a pH between 3.8 and 4.7 (DeGiusti et al., 1962). Acidic pH in the range of 3–4 would not have the ability to induce degradation of a synthetic polymer such as PS (Feng et al., 2011), but the pH associated with the enzymatic reaction and the digestive tract as presented, may play an important role in reducing the average particle size as this process occurs repeatedly. Under environmental conditions, where MP are subject to several abiotic forces that lead to aging and induce the embrittlement and degradation of the polymer matrix (Klein et al., 2017; Backhaus and Wagner, 2020), it is expected that the fragmentation process mediated by organisms could be facilitated.

Thus, it can be speculated that the changes in the surface and structure of the particles may have occurred due to the exposure to mechanical forces, gut enzymatic processes, the intestinal microbiome or even a combination of the three.

3.4. Mortality of amphipods due to PS microplastics exposure

The 7-day exposure period to PS-MP did not result in significant mortality of *H. azteca* ($p > 0.05$). Mortality was 6.67% at the concentrations 2700 and 5400 items/L compared to the negative control. No death was recorded at the lowest concentration (540 items/L). The results suggest that none of the PS concentrations tested exhibited toxicity, even the highest.

Overall, it is suggested that mortality is most often related to high concentrations of microplastics in the medium (Au et al., 2015; Gerhardt, 2020; Jeyavani et al., 2022b), and the lack of mortality observed in this experiment can be attributed to the realistic concentrations tested in the present study. Most of previous MP exposure experiments carried out on microcrustaceans *Gammarus* genus, a freshwater amphipod with

behavior similar to *H. azteca*, have not shown any effect on mortality. In exposure experiments by Weber et al. (2018) no sign of decreased survival or effect on the measured parameters were detected, the particles (0.8–4000 items/mL) initially ingested were continuously egested, but no accumulation in the digestive tract was documented. This result corroborates with the findings of the study conducted by Kuehr et al. (2022) on *H. azteca* to verify the bioaccumulation of nano and MPs, that did not show any evidence of bioaccumulation. On the other hand, despite Redondo-Hasselerharm et al. (2018) found no effect on survival rate (under a concentration up to 40% plastic in sediment dry weight) of *Gammarus pulex* and *H. azteca* a significant reduction in growth was recorded after exposure to environmentally relevant MP conditions.

4. Conclusions

To the best of our knowledge, this is the first study to demonstrate that the amphipod *H. azteca* can induce microplastic fragmentation in PS spheres in the presence of food. After the 7-day exposure period, the average sizes of MP PS were significantly smaller compared to the initial size. And the reduction particle size was constant as shown in timeline analysis. The reduction in particle size is a strong indication that this may be an important route for trophic transfer. Although particle ingestion was successfully observed, microplastics had no negative effect on *H. azteca* survival under the conditions tested, in contrast, exposure was sufficient to lead to oxidative stress. Due to the different bonds and chemical structures of the polymers that constitute microplastics, future studies should focus on investigating other polymer matrices, considering different morphologies and structural conditions, and other species of benthic organisms.

CRediT authorship contribution statement

Bárbara Rani-Borges: Conceptualization, Methodology, Formal analysis, Data curation, Investigation, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition. **Lucas Gonçalves Queiroz:** Methodology, Formal analysis, Data curation, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Caio César Achiles Prado:** Formal analysis, Data curation, Writing – original draft. **Eduardo Carmine de Melo:** Formal analysis. **Beatriz Rocha de Moraes:** Formal analysis. **Rômulo Augusto Ando:** Formal analysis, Funding acquisition. **Teresa Cristina Brazil de Paiva:** Formal analysis, Funding acquisition. **Marcelo Pompêo:** Supervision, Writing – review & editing, 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.

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