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Effects of PET microplastics on the freshwater crustacean *Daphnia similis* Claus, 1976

Efeitos dos microplásticos de PET no crustáceo de água doce *Daphnia similis* Claus, 1976

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Abstract: Aim: In this study, we investigated the effects of secondary PET microplastics (< 53 µm) on the *Daphnia similis* basic life-history parameters survival, age at first reproduction and total offspring number. We also analyzed *D. similis* enzymatic activity (superoxide dismutase, catalase and glutathione-S-transferase) at sub-effect concentrations. **Methods:** We performed acute and chronic toxicity tests using six PET microplastics concentrations (0, 10^2, 10^3, 10^4, 10^5 and 10^6 part. mL^{-1}). We also applied an exposure test to analyze superoxide dismutase, catalase and glutathione-S-transferase activities at sub-effect concentrations. **Results:** *D. similis* mortality increased (LC_{50} = 1 x 10^5 part. mL^{-1}), reproduction decreased (EC_{50} = 10^5 part. mL^{-1}) and time to first offspring was delayed by 5 days at the highest microplastic concentration after 21 days exposure. Neither mortality nor alterations in superoxide dismutase, catalase or glutathione-S-transferase activities were detected after 48 h exposure. Microplastics adhered to *D. similis* body appendages, causing altered swimming behavior. **Conclusions:** Lack of acute toxicity but occurrence of chronic effects serve as a warning for caution when concluding about microplastics non-toxicity in short-term tests. Microplastics had unexpected effects with important ecological implications. Our results contribute to fill the knowledge gaps on the effects of microplastic pollution on aquatic ecosystems, especially under long-term exposure.

Keywords: plastic; toxicity; polyethylene terephthalate; zooplankton.

Resumo: Objetivo: Neste estudo, nós investigamos os efeitos de microplásticos secundários de PET (< 53 µm) nos parâmetros básicos da história de vida de *Daphnia similis* sobrevivência,

idade na primeira reprodução e número total de descendentes. Nós também analisamos a atividade enzimática de *D. similis* (superóxido dismutase, catalase e glutationa-S-transferase) em concentrações de sub-efeito. **Métodos:** Realizamos testes toxicológicos agudos e crônicos usando seis concentrações de microplásticos PET (0, 10^2, 10^3, 10^4, 10^5 e 10^6 part. mL^{-1}). Também aplicamos um teste de exposição para analisar a atividade da superóxido dismutase, catalase e glutationa-S-transferase em concentrações de sub-efeito. **Resultados:** A mortalidade de *D. similis* aumentou (CL_{50} = 1 x 10^5 part. mL^{-1}), a reprodução reduziu (CE_{50} = 10^5 part. mL^{-1}) e o tempo para a primeira prole foi atrasado em 5 dias na maior concentração de microplástico após 21 dias de exposição. Não foram identificadas mortalidade nem alterações na atividade da superóxido dismutase, catalase ou glutationa-S-transferase após 48 h de exposição. Os microplásticos se aderiram aos apêndices corporais de *D. similis*, causando alterado comportamento natatório. **Conclusões:** A ausência de toxicidade aguda e a ocorrência de toxicidade crônica alertam para cautela ao concluir sobre a não toxicidade dos microplásticos em testes de curto prazo. Os microplásticos apresentaram efeitos inesperados com implicações ecológicas importantes. Nossos resultados contribuem para preencher as lacunas de conhecimento sobre os efeitos da poluição microplástica nos ecossistemas aquáticos, especialmente sob exposição a longo prazo.

Palavras-chave: plástico; toxicidade; polietileno tereftalato; zooplâncton.

1. Introduction

Environmental ubiquity of microplastics, plastic particles smaller than 5 mm, is currently one of the biggest challenges worldwide related to plastic pollution (Wagner et al., 2014; Hale et al., 2020). Microplastics pollution is not limited to cities but extends to remote regions, such as islands (Heskett et al., 2012), lakes in mountainous regions (Free et al., 2014), glaciers (Peeken et al., 2018) and even the deeper reaches of the Mariana Trench (Weston et al., 2020).

Microplastics enter aquatic ecosystems through industrial and household sewage discharge, poor solid waste management, surface runoff from urban areas (Wu et al., 2019) and even through atmospheric transport (Allen et al., 2019; Brahney et al., 2021). According to their origin, microplastics can be defined as primary – when manufactured as micro particles, or as secondary – when generated by fragmentation of larger materials such as packaging (Fahrenfeld et al., 2019). Secondary microplastics are the most common in aquatic ecosystems because of their continuous fragmentation (Fu & Wang, 2019).

Their origin and nature can influence microplastic toxicity (Ogonowski et al., 2016). Around 40% of plastics production is for the packaging segment (Plastics Europe, 2019), in which polyethylene terephthalate (PET) resin is one of the most consumed (Hahladakis et al., 2018). PET is also one of the least degradable plastics, which favors its persistence in the environment as secondary microplastics (Hahladakis et al., 2018). However, studies related to microplastics toxicity have focused on polystyrene and polyethylene microplastics (Jeong & Choi, 2019), while few studies have investigated effects of PET microplastic on aquatic organisms (Heindler et al., 2017; Weber et al., 2018; Parolini et al., 2020; Piccardo et al., 2020; Provenza et al., 2020).

Microplastics have the same size range as plankton components, which are food resources for a plethora of aquatic animal groups, such as zooplankton (Figueiredo & Vianna, 2018). Microplastic ingestion by zooplankton has been identified both in laboratory studies (Jaikumar et al., 2018; Kokalj et al., 2018) and natural environments (Kosore et al., 2018; Jones-Williams et al., 2020). Potential toxic effects caused by microplastics ingestion on zooplankton is an ecological threat (Ma et al., 2020), especially because of the key position zooplankton occupy in aquatic food webs (Maar et al., 2004; Chew et al., 2012).

The main effects reported in diverse animal groups after microplastic exposure include decreased growth and feeding, increased mortality and reproductive failure, caused mainly by the production of reactive oxygen species (ROS) (Jeong & Choi, 2019). Increased mortality, longer interval between reproductions and a smaller number of neonates were reported in the freshwater planktonic specie *Daphnia magna* Straus, 1820 after a 21-day exposure to polyethylene at 10^5 part. mL^{-1}. On the other hand, Eltemsah & Bøhn (2019) evaluated the effects of polystyrene microspheres to *Daphnia magna*, in a chronic trial prolonged to 77 days, and found a slight reduction in the number of neonates and in parental body weight only at the higher concentrations (30, 50 and 100 $mg.mL^{-1}$) and after 45 days exposure.

Tang et al. (2019) suggest that *D. magna* oxidative defense, energy production and extracellular transport are significantly affected by exposure to microplastics. Microplastics exposure can also alter an animal's oxidative state, without any physical

signs of change in its health status, as reported in the Manila clam, *Ruditapes philippinarum* (A. Adams & Revee, 1850) (Parolini et al., 2020).

The genus *Daphnia* is one of the main representatives of freshwater zooplankton and is often used in microplastics bioassays (Canniff & Hoang, 2018; Ebert, 2022; Jaikumar et al., 2018; Liu et al., 2020). However, only one study investigated microplastic toxicity to *Daphnia similis* Claus 1976 until now (Jeyavani et al., 2023). Given that some microplastic toxic effects are species-specific (Jaikumar et al., 2018), the investigation of microplastic effects on this species is an important contribution to the literature.

This study investigated the acute and chronic toxicity of secondary PET microplastics (< 53 µm) on the freshwater *Cladocera Daphnia similis* and analyzed changes in *D. similis* oxidative state at sub-effect PET microplastics concentrations. We hypothesized that exposure to PET microplastics decreases *D. similis* survival and reproduction and that *D. similis* enzymatic activity is altered, even without any evident toxic effects.

2. Materials and Methods

2.1. D. similis maintenance

D. similis were maintained according to the Associação Brasileira de Normas Técnicas guidelines (Brazilian Association of Technical Standards) ABNT NBR 37 12713:2016 (ABNT, 2016). *D. similis* were raised in 2 L beakers filled with culture medium at pH 7.0 - 7.2 and 44 mg $CaCO_3$ L^{-1} hardness, held at 22 ºC, under a 12h light/dark photoperiod (average illuminance of 900 lux). Culture medium was changed three times a week, when organisms were fed with a mixture of *Raphidocelis subcapitata* (Korshikov) Nygaard (10^6 cells per organism) and TetraMin Plus fish feed solution (20 µL per organism).

2.2. Microplastics and stock solution preparation

Pellets of transparent PET bottles were supplied by a recycling company. Pellets ranging from 1 mm to 2 mm were generated by post-consumer recycling process. Pellets were ground in a Super Macro Tecnal mill to obtain smaller, heterogeneous and irregularly shaped plastic pieces. Milled material was sieved through a stainless-steel sieve (53 µm mesh), resulting in secondary PET microplastics < 53 µm in size. Minimal size of microplastics was analyzed by vacuum filtration, using glass fiber filters with 8, 1.2 and 0.45 µm mesh. Both 1.2 and 0.45 µm filters were analyzed under a microscope. No particles were identified in the 0.45 µm filter, so that 1.2 µm was considered as an approximate lower limit of microplastic size.

A stock solution of 10^6 particles per mL was prepared by mixing 2.9 g of microplastics in 1 L of culture water. Microplastic particles per volume were counted using a Neubauer chamber, using the same methodology adopted for cell counting.

2.3. Microplastics settling velocity

Settling velocities were determined following Khatmullina & Isachenko (2017) with minor modifications. For this, three sink experiments were conducted in a glass graduated cylinder filled to 29 cm with a deionized water column. Then, microplastic particles (~ 0.01g) were placed approximately 1 cm below the water surface to avoid a restriction of surface water tension. Afterward, the particles were let fall freely and the time the particle took to cover 16.4 cm (sinking distance) was made with a stopwatch. The settling velocity was calculated as the ratio of sinking distance to the time of fall.

2.4. Acute toxicity experiment

D. similis neonates (< 24 h old) were exposed to 6 concentrations of PET microplastics (0, 10^2, 10^3, 10^4, 10^5 and 10^6 part. mL^{-1}). Stock solution dilutions were prepared immediately before the start of the assay. Each treatment (concentration) included 4 replicates (25 mL Erlenmeyer), containing 10 mL of test solution and 5 neonates, resulting in 20 neonates per treatment. After 48 h of exposure, daphnids were inspected for mortality and microplastic ingestion was conferred through observation of their digestive tracts using optical microscopy. Mortality results were expressed as the percentage of dead animals per treatment. The assay was performed without test solution renewal or feeding, in accordance to ABNT NBR 12713: 2016 guidelines (ABNT, 2016).

2.5. Chronic toxicity assay

D. similis neonates (< 24 h old) were exposed to the same 6 concentrations used in the acute toxicity assay. The chronic assay was performed according to the OECD 21-day *Daphnia* reproduction test method (OECD, 2012). Test solution renewal and feeding were carried out every 48 hours, for the duration of the assay. Effects of chronic exposure on the *D. similis* basic life-history parameters survival, age at first reproduction and total offspring number were evaluated. Microplastic ingestion by *D. similis*

was conferred through visual observation of the digestive tract using optical microscopy.

2.6. Exposure to oxidative state analyzes and tissue preparation

Oxidative enzymatic activity analyses were carried out to verify if any damage was caused to *D. similis,* even in the absence of mortality after 48 h of exposure. Five microplastic concentrations (0, 10^2, 10^3, 10^4 and 10^5 part. mL^{-1}) were selected based on results of acute and chronic toxicity tests. Each treatment included 4 replicates (250 mL beakers), containing 150 mL of test solution and 40 *D. similis* adults (7 days old). Adults were exposed to microplastics for 48 hours.

After exposure, body mass of the organism pool from each replicate was weighed and organisms were kept frozen at -80ºC until further analysis. Homogenates of approximately 40 mg of *D. similis* (N = 40) tissue were prepared in phosphate buffer (0.2 M, pH 7.0 with EDTA) at a dilution ratio of 100 µL of buffer for each 10 mg of tissue. Samples were crushed for 20 s with a homogenizer and subsequently centrifuged at 12,000 revolutions per minute (rpm) for 10 min at 4 °C. Sample supernatants were used for superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) activity determinations.

2.7. SOD, CAT and GST activity determination

SOD activity was determined based on the reduction of superoxide (O_2^-) and hydrogen peroxide decreasing the autooxidation of pyrogallol (Dieterich et al., 2000). The reaction mixture contained 99 µL potassium phosphate buffer (5 mmol L^{-1}, pH 8.0) and 30 µL sample to which 15 µL pyrogallol (100 µmol L^{-1}) were added. Reaction mixture absorbance was measured at 570 nm. SOD activity was calculated as units (U) per milligram of protein, with one U of SOD defined as the amount that inhibited pyrogallol autoxidation by 50%. Duplicates of negative and positive controls were prepared without and with pyrogallol, respectively.

CAT activity was determined by the method of Hadwan & Abed (2015), using hydrogen peroxide (H_2O_2) as substrate. 100 µL H_2O_2 (20 mM) were pipetted into 5 µL of sample. After 3 min, 150 µL ammonium molybdate (32.4 mmol L^{-1}) were added to stop the reaction. Negative controls were prepared by replacing H_2O_2 with sodium and potassium phosphate pH buffer (50 mM, pH 7.0). Sample absorbance was read at 374 nm. CAT values were extrapolated from a standard curve was constructed using serial dilutions of bovine albumin. Sample test values were subtracted from the control test values to obtain concentrations and results were expressed as units of CAT per milligram of protein ($U.mg^{-1}$).

Glutathione (GSH) conjugation to 1-chloro-2, 4-dinitrobenzene (CDNB) was monitored spectrophotometrically at 340 nm for detection of GST enzyme activity (Habig et al., 1974), using a CDNB molar extinction coefficient, ε 340 = 9.6 $mmol.L^{-1}.cm^{-1}$. One unit of GST was defined as the amount of enzyme that catalyzed the formation of 1 µmol GSH min^{-1}. mL^{-1}.

2.8. Statistical analysis

Data were submitted to unifactorial analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons, at a 5% level of significance. When normality was not identified, data were analyzed using the Kruskal-Wallis test. Association between mortality and microplastics concentration was evaluated using the Cochran-Armitage test for trend. PET microplastic concentrations that caused 50% death (LC_{50}) and altered reproduction (EC_{50}) were calculated at a 5% level of significance using the Spearman-Karber method. Results were expressed as mean ± standard deviation (Mean ± Sd).

3. Results

3.1. Microplastics settling velocity

The microplastic settling velocity test and their concentrations at the settling time were depicted in Table 1.

3.2. Toxicity tests

Microplastic ingestion was confirmed after both acute and chronic exposure (Figure 1).

No acute toxicity was verified after 48 hours exposure, with less than 10% mortality at all microplastic concentrations tested, and therefore, a 48-hour LC_{50} could not be calculated (ABNT, 2016).

Table 1. Microplastics PET settling velocities.

Experiment	Time (s)	Settling velocity
I	13.32	1.23
II	13.02	1.26
III	9.37	1.75
Average ± standard deviation, n = 3		**1.41 ± 0.22 cm s^{-1}**

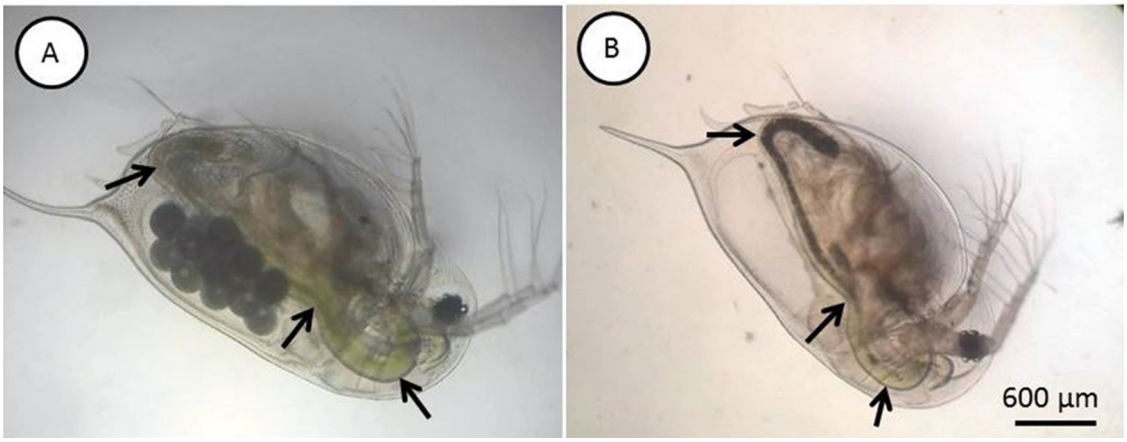

Figure 1. Adult *Daphnia similis* digestive tubes by optical microscopy. (A) Individual from control; digestive tube almost empty, but with green aspect from the algae food; (B) Individual from 10^5 part.mL^{-1} treatment; digestive tube fully of PET microplastics aggregated (black aspect). Arrows indicate the digestive tract.

D. similis mortality increased with microplastic at the highest microplastic concentration (Figure 2), with a LC_{50} of 1 x 10^5 part. mL^{-1}.

During chronic exposure, neonates of dead parents were considered to evaluate microplastic effects on *D. similis* reproduction, due to dose-response detection for deaths (OECD, 2012). The microplastics concentration that altered reproduction by 50% was 2.9 x 10^5 part. mL^{-1}, with a reduction in total offspring from 54.55 (± 16.88) in the control to 10.20 (± 14.83) at the highest microplastics concentration (Figure 3A). Time to first offspring was delayed, ranging from 8.78 (± 1.39) days in the control to 13.60 (± 2.97) days at the highest microplastic concentration (Figure 3B).

Besides altered life history parameters, we also observed a visible physical effect of microplastics fixation on *D. similis* thoracic appendages and antennae (Figure 4). These observations were very often accompanied by an altered *D. similis* swimming pattern.

3.2.1. Superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) activities

No altered patterns were observed in SOD and GST activities, although a trend of increased CAT activity was observed with the increase in microplastic concentration (Table 2). No statistical differences in enzyme activities at different concentrations were found ($p > 0.05$, Kruskal Wallis) after 48h exposure, indicating no induction of oxidative stress in *D. similis* under acute exposure PET microplastics.

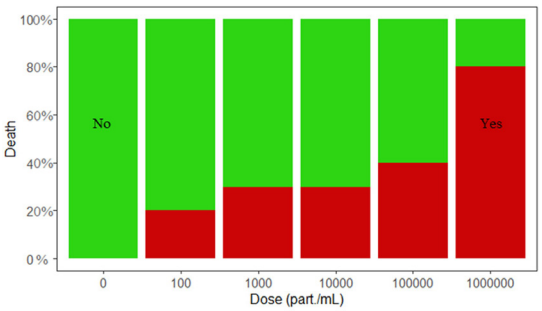

Figure 2. *Daphnia similis* mortality (%) upon 21-day exposure to varying PET microplastic concentrations (N = 10 per treatment).

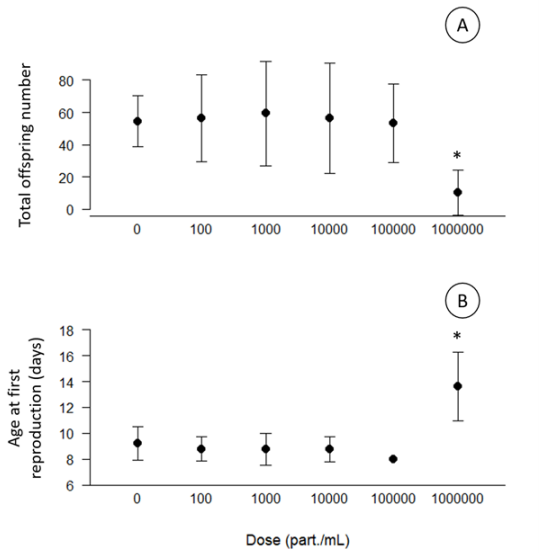

Figure 3. *Daphnia similis* (A) total offspring number and (B) age at first reproduction after, 21 days exposure to increasing polyethylene terephthalate (PET) concentrations (N = 10 per treatment). * Significant difference from control (dose = 0), $p < 0.05$.

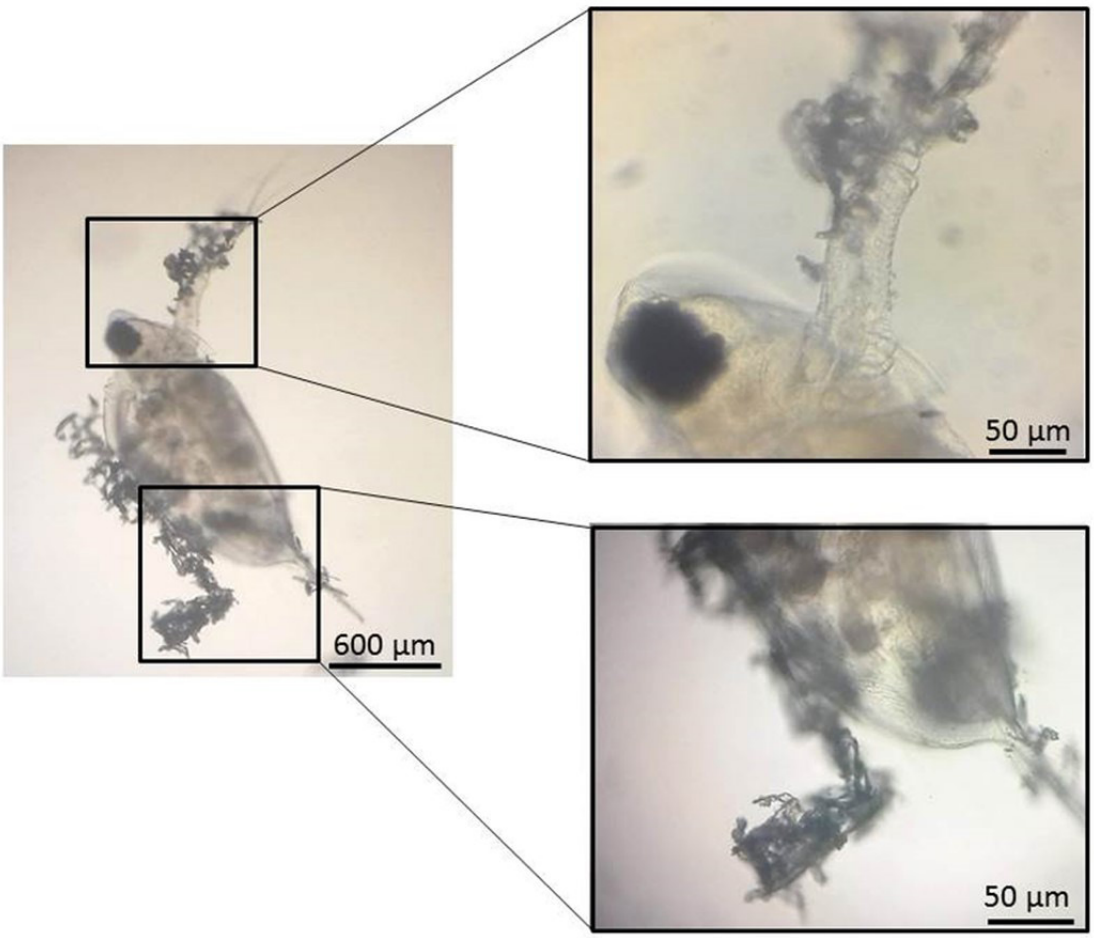

Figure 4. Polyethylene terephthalate (PET) microplastics attached to *Daphnia similis* thoracic appendages and antennae.

Table 2. *Daphnia similis* enzymatic activity after 48-hour exposure to different polyethylene terephthalate (PET) microparticle concentrations. SOD: superoxide dismutase; CAT: catalase; GST: glutathione-S-transferase.

Concentration (part. mL^{-1})	SOD ($U.mg^{-1}$ of protein)	CAT ($U.mg^{-1}$ of protein)	GST ($\mu mol.min^{-1}.mL^{-1}$)
0	2.16 ± 0.57	385.23 ± 74.27	2066.04 ± 812.61
10^2	2.10 ± 0.83	442.00 ± 226.23	2066.04 ± 582.76
10^3	2.86 ± 0.62	510.00 ± 104.88	1744.85 ± 426.57
10^4	2.48 ± 0.70	602.96 ± 208.67	1710.12 ± 163.79
10^5	3.13 ± 1.61	728.39 ± 419.02	2083.40 ± 830.46

4. Discussion

Polyethylene terephthalate (PET) microplastics inhibit survival and reproduction in the freshwater crustacean *D. similis*, but only at high concentrations (> 10^5 part. mL^{-1}) and long-term exposure. However, a significant dose response was observed for mortality at chronic exposure levels.

The results obtained are similar to those found by Ogonowski et al. (2016), who observed reduced *D. magna* mortality and reproduction only at very high secondary polyethylene concentrations (10^5 part.mL^{-1}) after 21 days exposure. However, the maximum concentration tested in that study was one order of magnitude less than in this study, indicating a less pronounced toxic effect in our study. At 10^6 part.mL^{-1} PET secondary microplastics, we also observed a delay of 4.82 days to *D. similis* first reproduction, while at 10^5 part. mL^{-1} secondary polyethylene, Ogonowski et al. (2016) reported no significant effect on *D. magna* age at first reproduction.

Several studies demonstrated that microplastics toxicity is size and concentration-dependent, with

smaller microplastics at higher concentrations causing higher toxicity (Jeong et al., 2016; Wu et al., 2021; Zhang et al., 2022). In a previous study, *D. magna* was exposed to large PET fibers at 1 part. mL^{-1} and a mortality rate up to 40% was reached after 48 hours (Jemec et al., 2016). That result differs from those of the present study, in which a mortality rate lower than 10% was observed after 48h exposure to microplastic concentrations up to 10^6 part. mL^{-1}. Thus, large PET fibers at extremely low concentrations presented higher toxicity in that study than smaller PET microplastics (1.2 – 53 μm) at high concentrations in this study. Differences in methodologies might explain the differences in results, since, besides different microplastic shapes, different test species were used in our studies. Different toxicities for the same type of plastic depending on the particle shape, as well species-specific effects, have been widely reported in literature (Ogonowski et al., 2016; Canniff & Hoang, 2018; Jaikumar et al., 2018).

The absence of an acute effect, but occurrence of a chronic effect, as found in this study, has been previously reported in studies on microplastics. For example, no acute but chronic toxicity of different microplastic types were reported to *D. magna*, *Daphnia pulex* Leydig, 1860 and *Ceriodaphnia dubia* Richard, 1894, whose survival was strongly time-dependent and substantially more severe after 48 h exposure (Jaikumar et al., 2018). Similarly, no acute toxicity of polystyrene microplastics to *D. magna* within 48 h, but slightly increased mortality, reduced growth and stimulation of early reproduction at the cost of later reproduction within 90 days exposure were reported by Eltemsah & Bøhn (2019). All these results should raise awareness of the potential negative effects of chronic exposure to microplastics in the environment.

D. similis oxidative status after acute exposure to microplastics was investigated as a possible non detectable adverse effect or a possible trigger mechanism to observed effects. In contrast to our expectations, oxidative stress enzyme activities were not affected by exposure up to 10^5 part. mL^{-1}, indicating that PET microplastics of 1.2 – 53 μm do not alter enzymatic activity in *D. similis*. Increased activities of glutathione peroxidase (GPx), glutathione reduced (GR), GST and SOD were reported as size-dependent in the rotifer *Brachionus koreanus* Hwang, Dahms, Park & Lee, 2013 and in the copepod *Paracyclopina nana* Smirnov, 1935 exposed to microbeads of different sizes for 24 h (Jeong et al., 2016, 2017). In *Paracyclopina nana*, polystyrene nanoplastics (0.05 μm) were capable of permeating cell membranes and had higher bioavailability than microplastic particles (0.5 and 6 μm), resulting in reduced growth and reproductive rates in response to cellular damage. In our study, we used a heterogenous suspension of 1.2 – 53 μm PET microplastics, particles too large to be translocated between cells. Therefore, the chronic effects observed may have resulted from a direct physical effect of the microplastics, and not from prior generation of ROS.

Physical effects related to microplastics ingestion include blockages throughout the digestive system (Wright et al., 2013). This blockage triggers secondary adverse effects, such as false satiety with reduced eating, reproductive failure and death (Galgani et al., 2010). Thus, the dose-response for death observed in the present study may have occurred as a consequence of increasing obstruction of the *D. similis* digestive tract with increasing microplastics concentration, with more pronounced effects at the highest concentration.

Not only ingestion, but also microplastics fixation to *D. similis* appendages and antennae may also have caused adverse effects, a phenomenon reported by other authors (Cole et al., 2015; Cui et al., 2017; Ziajahromi et al., 2017). *D. similis* uses body appendages to direct water to the mouth, enabling the filtration process (ABNT, 2016). Increased mortality with increasing microplastics concentrations may have been caused by impairment of *D. similis* appendages, leading to decreased food intake. Although food ingestion was not assessed in the present study, Ogonowski et al. (2016) identified less carbon absorption in organisms exposed to microplastics compared to the control group. Similarly, Tang et al. (2019) identified that exposure to polystyrene microparticles led to a significant increase in *D. magna* arginine kinase (AK) expression, an enzyme involved in energy production, which was interpreted by the authors as an attempt to compensate an energy source limitation.

Microplastics fixation to appendages and antennae has others important implications, beyond impaired food intake. *D. similis* uses antennae for swimming and very often showed an altered swimming pattern when microplastics adhered to their antennae and tails. A similar result was reported for *C. dubia*, which showed an inability to swim when exposed to polyester

fibers (Ziajahromi et al., 2017). The energy loss caused by weakened swimming ability led to lower growth and lower reproduction in the organisms (Ziajahromi et al., 2017). Altered swimming patterns can slow down the affected organisms and make them more vulnerable to predators (Vaz et al., 2021). These results indicate that microplastics may have ecological implications beyond the effects commonly investigated in toxicity assays.

Parthenogenetic reproduction in *Daphnia* is controlled, among other factors, by estrogen hormones, which function in embryonic development and egg reserve formation (Xu et al., 2020). Some plastics additives have the ability to bind to estrogen receptors, acting as agonists of estrogen and resulting in estrogenicity (Yang et al., 2011). In this study, plastic leachate was not analyzed. However, previous studies showed that PET extracts presented low or no estrogenic activity (Zimmermann et al. 2019) and nonexistent or very low PET additive migration to water (Li et al., 2016). Thus, we suggest that the reduction in total offspring number and delay on age at first reproduction observed in this study were a result of physical effects of microplastics, triggered by the processes discussed previously.

Sedimentation of PET microplastic particles, which are denser than water, occurred within a few minutes in the exposure assays, with smaller particles remaining in the water column for a longer time. Sedimentation reduced the concentration in the water column, at a rate of approximately 10^3 part.h^{-1} (Table 1). Thus, microplastic concentration depended not only on the exposure concentration, but also on which region of the test compartment *D. similis* inhabited during the assay. Even in studies performed with lower density plastics, such as polyethylene, microparticle sedimentation has been observed, because of their hydrophobicity and tendency to agglomerate. Some authors used surfactants to facilitate microplastic dispersion in the water column (Ogonowski et al., 2016; Ziajahromi et al., 2017; Jaikumar et al., 2018). However, following OECD standard recommendations, we opted not to use these (potentially toxic) chemicals.

The microplastics concentrations tested were higher than those reported in the environment. However, it is important to note that most environmental studies analyze microplastics larger than those used in this study (Fu & Wang, 2019; Wu et al., 2019) and that, because of the fragmentation process, it is expected that smaller microplastics occur in the environment at higher concentrations than larger ones (Barnes et al., 2009; Materić et al., 2022). Thus, the results observed contribute to comprehend microplastic interactions and effects on biota, possibly in actual, as well as in future scenarios.

5. Conclusion

Secondary PET microplastics presented no acute toxicity to *D. similis*, but increased mortality with increased microplastic concentration at chronic exposure levels. Furthermore, a reduction in number of total offspring and a delay to first reproduction were observed after 21 days exposure to 10^6 part.mL^{-1}. Absence of an acute effect and presence of a chronic effect serve as a warning for caution when interpreting results and drawing conclusions from short-term experiments, since in the environment species are subject to chronic exposure. Altered behavior observed in the organisms due to microplastics adherence on their bodies should be expected to have important ecological implications. Despite the higher microplastics concentrations used compared to environmentally relevant concentrations, the *in vitro* results are not irrelevant, since microparticles tend to increase in number in the environment through the fragmentation process. We recommend that future studies evaluate microplastic sedimentation rates, since sedimentation may reduce exposure concentrations over time. The non-lethal impacts observed in this study contribute to fill the knowledge gaps on the effects of microplastic pollution on aquatic ecosystems.

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