Biological responses of Chironomus sancticaroli to exposure to naturally aged PP microplastics under realistic concentrations

Bárbara Rani-Borges (barbara.rani-borges@unesp.br)
São Paulo State University
Lucas Gonçalves Queiroz
University of São Paulo, USP
Caio César Achiles Prado
University of São Paulo, USP
Beatriz Rocha Moraes
University of São Paulo, USP
Rômulo Augusto Ando
University of São Paulo, USP
Teresa Cristina Brazil Paiva
University of São Paulo, USP
Marcelo Pompêo
University of São Paulo, USP

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Abstract

Microplastic (MP) is yet another form of chronic anthropogenic contribution to the environment. MP are plastic particles (< 5 mm) that have been widely found in the most diverse natural environments, but their real impacts on ecosystems are still under investigation. Here, we studied the toxicity of naturally aged secondary polypropylene (PP) MP after constant exposure to ultraviolet radiation (26 µm) to larvae of *Chironomus sancticaroli,* a dipteran species. The concentrations tested were 13.5; 67.5; and 135 items.g^{-1} of dry sediment. *C. sancticaroli* organisms were investigated for fragment ingestion, mortality and changes in enzymatic biomarkers after 144 hours of exposure. The organisms were able to ingest MP from the first 48 hours, and the amount of items internalized was dose-dependent and time-dependent. Overall, the results show that mortality was low, being significant at the lowest and highest concentrations (13.5 and 135 items.g^{-1}). Regarding changes in enzyme markers, after 144 hours MDA and CAT activities were both significantly altered (increased and reduced, respectively), while SOD and GST levels were unchanged. In the present study, naturally aged polypropylene MP induced biochemical toxicity in *C. sancticaroli* larvae, with toxicity being higher according to exposure time and particle concentration.

1 Introduction

Due to low cost and wide applicability, global plastics production has increased rapidly, reaching 367 million tonnes in 2020, not including fiber plastics (Plastics Europe 2021). Due to its intense use worldwide and lack of proper management system, plastic has become a pollution problem of global proportions (Geyer et al. 2017; Horton and Dixon 2017). The investigation of microplastics (MP) as contaminants of environmental matrices can be considered a relatively new area of study, although knowledge about the presence of plastic particles in aquatic environments was initially reported since the early 1970s (Carpenter and Smith 1972). As a rule, MP are particles up to 5 mm in diameter (Courtney et al. 2009), and can also be divided into small (< 1 mm) and large (1 to 5 mm) MP (Eriksen et al. 2014; Hanvey et al. 2017).

Although MP has been widely reported in aquatic ecosystems around the world, demonstrating its occurrence in water bodies (Cincinelli et al. 2017; Luo et al. 2019) and its toxic potential for certain groups of organisms, such as annelids, crustaceans and fish (Doyle et al. 2022; Qiao et al. 2022), there is a great lack of knowledge regarding other classes. So far, Diptera species have been superficially studied as having neglected toxic effects due to exposure to MP. Diptera is an order of insects that have at least one of their life cycle stages in aquatic environments. This group of organisms is considered extremely rich, comprising approximately 158 families and more than 159,000 recognized species (Ibáñez-Bernal et al. 2020), of which 46,000 are aquatic species. In addition, they are the only aquatic insects that colonize all continents, including Antarctica (Adler and Courtney 2019).

Chironomid larvae are considered models in toxicity tests due to their wide distribution in aquatic environments, ease of cultivation in the laboratory, short life cycle and their biological characteristics,

which confer the ability to adapt to adverse environments (OECD/ OECD, 2010 Rosa et al. 2014; Serra et al. 2017). The species *Chironomus sancticaroli* has been widely used in the literature as a bioindicator of environmental quality with several pollutants and chemical compounds, including MP (Pinto et al. 2021; Rebechi et al. 2021; Palacio-Cortés et al. 2022). The life cycle of *C. sancticaroli* is characterized by four stages: egg, larva, pupa and adult (aerial). Of these, the first three are aquatic stages with benthic habits (Strixino and Strixino 1982). Due to their ecological role, Chironomidae larvae are an important biological representative of the benthic macrofauna and it is essential to understand their behavior and interaction with the various pollutants present in aquatic ecosystems.

MP are subject to several environmental factors that lead to chain breakage and plastic degradation. Among the environmental conditions that can act in this process, it is possible to mention temperature, weathering, intensity of ultraviolet radiation (UV), winds, physical friction, salinity and pH (Antunes et al. 2013, Liu et al. 2016, Wagner and Lambert 2017, Torres et al. 2020). Aged MP present increased toxicity due to the release of monomers, additives and compounds generated by the reactions of these degradative processes (Hermabessiere et al. 2017, Zhang et al. 2019), and the increase in the porosity of the particles, which will facilitate the sorption of chemical compounds present in the environment. In toxicity tests, the most studied particles refer to primary or artificially aged MP, due to experimental practicality. However, even though the environmental distribution and toxicity of MP have already been investigated, only limited information is available for the environmental transformation of this pollutant in the laboratory. Therefore, it is necessary to investigate the phototransformation of MP under natural radiation to understand how the aging process can influence the potential risks of MP to biota.

Considering the scenario presented, the present study aimed to study the effects of exposure of *C. sancticaroli* larvae to naturally aged polypropylene MP (PP; PP-MP) through diet. In order to establish a pattern of response at the cellular and/or individual level, the organisms were evaluated for PP-MP intake, changes in oxidative stress markers and mortality after 6 days of exposure.

2 Material And Methods

2.1 Microplastic

The MP used in the tests was composed of polypropylene (PP) of bluish color, secondary, from the lid of a storage container that was exposed to ultraviolet radiation for an indefinite period, until the fragmentation of the material was visible. The plastic underwent high-energy milling and the fragments were passed through a system of metal sieves in a column coupled to a sieve (Godoy et al. 2019; Stock et al. 2019) with several mesh openings until it was obtained the size of 26 µm. To confirm the particle size, 100 particles were measured under a stereomicroscope (Zeiss Discovery V12). The particles were stained with the fluorescent dye Nile Red (99% pure, INLAB, Brazil), at a concentration of 300 $mg.L^{-1}$ (Prata et al. 2019). The stained particles were suspended in ultrapure water and the concentration was determined by manually counting 100 µL (n = 5) under a stereomicroscope (10 µL = 135 ± 12 items). The solution was kept at 4°C in the dark until used.

The shape of the particles was observed and characterized by a stereomicroscope (Zeiss Discovery V12) and by an inverted fluorescence microscope (Leica DMi8) and the chemical composition was performed by Fourier transform infrared spectroscopy (FTIR). The FTIR spectra were measured in a Bruker spectrometer, model Alpha, in the region of 400–4000 cm^{-1}, with a standard KBr beam splitter and a high sensitivity DLATGS detector. The spectra were recorded with the ATR (Attenuated Total Reflection): ATR Platinum module, 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 Cultivation of *C. sancticaroli*

The larvae of *C. sancticaroli* used were obtained from a continuous culture maintained in the laboratory (OECD 2011) by transferring newly laid eggs to glass vessels containing a thin layer of sediment and mineral water under constant aeration. The vessel containing the spawns were kept in an incubator at 25°C with a photoperiod of 12:12 h (light:dark) and monitored daily to obtain organisms aged 6 days after hatching for the experiment.

2.3 Exposure of organisms to PP microplastics

C. sancticaroli larvae were exposed to 3 different concentrations of PP-MP, being 13.5; 67.5; and 135 $items.g^{-1}$ of dry sediment. The experiment had a total duration of 144 hours and was carried out in glass flasks. Each treatment consisted of six replicates and 10 g of calcined sediment, 200 mL of culture water and 1000 µL of a suspension used to feed the larvae in the culture (5 g of $Tetramin.L^{-1}$) were added to each replica. Food was offered every 48 hours. The containers were kept at the same temperature (25 ± 1°C) and photoperiod (12:12, light:dark) conditions as the culture and under constant aeration (1 $bubble.s^{-1}$).

2.4 Ingestion of PP microplastics by *C. sancticaroli*

Every 48 hours, one larva was removed from each replicate (n = 6 per treatment) and fixed in 70% alcohol to assess PP-MP ingestion. The presence of particles inside the digestive tract of the larvae was evaluated using inverted fluorescence microscopy (Leica DMi8).

2.5 Biomarkers of enzyme activity

At the end of the experiment, larvae of each replica were removed from the container and used for evaluation of potential changes in biomarkers of enzymatic activity (n = 9 for enzymatic reactions; n = 5 for malonaldehyde analysis). From these organisms, a homogenate (1:9, w/v) was prepared by steeping in cold potassium phosphate buffer (pH 7.4). The homogenates were centrifuged at 2000 g for 20 min at 4°C, and the supernatants were collected to determine the enzymatic activity of glutathione S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD) and malonaldehyde (MDA) levels.

Protein contents were determined by the Bradford method using bovine serum albumin (BSA) as a standard (Bradford, 1976). Each enzyme activity was determined three times using the same homogenate and the results used to calculate the specific activity of GST, CAT, SOD and MDA.

2.5.1 Glutathione S-transferase (GST)

The activity of glutathione S-transferase (GST) was adapted from the method proposed by Habig et al. (1974). Assays were conducted in triplicate using 100 mM potassium phosphate buffer (pH 6.5), 1.0 mM EDTA, 9.5 mM reduced glutathione (GSH), 1.0 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and 10.0 µL homogenate. CDNB was used as a substrate for the conversion reaction of GSH into glutathione thiolate anion (GS–), through the GST enzyme. The formation of the S-(2,4-dinitrophenyl) glutathione conjugate was monitored to increase the absorbance at 340 nm for 5 min in the UV-VIS spectrometer. The molar extinction coefficient of CDNB was 9.6 $mM^{-1}.cm^{-1}$.

2.5.2 Catalase (CAT)

Catalase (CAT) activity was determined following the method described by Aebi (1984). Tests were conducted using 100 mM potassium phosphate buffer (7.0), 20.0 mM hydrogen peroxide (H_2O_2) and 10.0 µL of homogenate. Activity was monitored by consumption of H_2O_2 resulting in a decline in absorbance at 240 nm for 3 min in the UV-VIS spectrometer. The molar extinction coefficient for H_2O_2 was 40.0 $mM^{-1}.cm^{-1}$. The enzymatic activity was expressed from the consumption of 1 mmol of H_2O_2 min^{-1} mg of $protein^{-1}$.

2.5.3 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was analyzed by the reaction of pyrogallic acid with the sample, observed at 420 nm. 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 subsequently homogenized vigorously for 20 s. The assays were incubated for 30 min in the dark at 25ºC. After incubation, the oxidation reaction was stopped with the addition of 65 µL of 1N HCl. The same preparation was performed for the blank, using 60 µL of 100 mM potassium phosphate buffer pH 6.5. SOD activity was determined by the ability to inhibit the reduction of pyrogallol by superoxide radicals by 50% expressed as U/SOD.

2.5.4 Malonaldehyde (MDA)

Lipid peroxidation damage was evaluated through MDA levels, as described by Campos et al. (2014), with adaptations to better fit our samples. 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, organisms of *C. sancticaroli* (n = 5) were added and macerated with 400 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 ± 1ºC for 45 min. After being cooled in an ice bath, the samples were centrifuged at 3.000 rpm for 5 min at 25°C and read at a wavelength of 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 carried out for the standard control, adding 500 µL of 4.5 mM 1,1,3,3-tetraethoxypropane (TEP) and 1 mL of 0.4% TBA. Results were expressed as $nmol.mL^{-1}$ of MDA.

2.6 Statistical analysis

Data were expressed as mean ± standard deviation (SD). All statistical analyzes were performed using Minitab v.14 software. Data normality was determined by Anderson-Darling. Significant differences between treatment and control groups were analyzed using Dunnett's test ($p < 0.05$).

3 Results And Discussion

3.1 Characterization of PP-MP

PP-MP that was fractionated from the lid of a naturally photoaged container to a size of 26 µm and fluorescence stained with Nile red was visualized under a fluorescence microscope (Fig. 1). The irregular morphology and expected size of the particles were confirmed. The measurement of 100 particles under a stereomicroscope showed that the maximum size of PP-MP was 46.3 µm and the minimum size was 17.1 µm, while the mean diameter was 26 ± 5.2 µm.

FTIR analyzes showed that the MP samples were treated as PP particles (Fig. 2). After aging, the content of carbonyl groups is expected to increase compared to virgin materials, which characterizes oxidative degradation (Müller et al. 2018). This change in the polymer matrix can be observed by the presence of peaks 1714 cm^{-1} which correspond to carbonyl groups (C = O) (Romano et al. 2017).

3.2 Ingestion of microplastics

The number of PP-MP ingested by larvae was higher according to the exposure time. Thus, larvae exposed to the lowest concentration (13.5 $items.g^{-1}$) ingested an average of 1.0 to 2.5 items of PP-MP between 48 and 144 hours. At concentrations of 65.2 and 135 $items.g^{-1}$, the larvae ingested from 1.3 to 2.7 and from 2.8 to 4.0 PP-MP between 48 and 144 hours, respectively. The number of ingested particles was also proportional to the increase of PP-MP concentration in the sediments in all treatments (Table 1).

Table 1
Ingestion of aged polypropylene microplastics (PP-MP). Number of PP-MP particles present in the gastrointestinal tract of individuals of *Chironomus sancticaroli* (n = 6) after 48, 96 and 144 hours under three exposure conditions. The number of MP particles is given by the average number of MP per treatment ± SD.

Ingestion			
Treatment	48h	96h	144h
C1: 13.5 items.g^{-1}	1.0 ± 0.6	2.2 ± 1.2	2.5 ± 0.8
C2: 67.5 items.g^{-1}	1.3 ± 1.2	2.5 ± 1.4	2.7 ± 0.8
C3: 135 items.g^{-1}	2.8 ± 2.7	4.0 ± 0.9	4.0 ± 1.1

Like other benthic organisms, *Chironomus* sp. are opportunistic omnivores that have a diet based on particulate organic matter (Armitage et al. 1995). In the environment, these organisms ingest a wide variety of food items without much selectivity (Cummins and Klug 1979). Therefore, eating habits make this genus susceptible to solid pollutants present in sediments, such as MP. Several studies have already reported the ability to ingest MP particles by different species of *Chironomus*, both under controlled laboratory conditions (Scherer et al. 2017, 2019) and in environmental conditions (Nel et al. 2018). Images of *C. sancticaroli* larvae from the present study with particles in the gastrointestinal tract were recorded and can be seen in Fig. 3.

Despite MP ingestion being one of the prerequisites to induce toxicity in exposed organisms, the low volume of studies with this genus does not allow definitive outcomes to be inferred on the consequences of such interaction. Still, MP ingestion by chironomids is thought to trigger gastrointestinal tract obstruction, which can lead to changes in food intake or nutrient absorption (Avio et al. 2015; Nel et al. 2018; Ziajahromi et al. 2018; Ziajahromi et al. 2018; Silva et al. 2019). In more extensively studied groups of organisms, such as *Danio rerio*, some studies have also reported that exposure to MP can lead to neurotoxicity and behavioral changes (Chen et al. 2020; Wan et al. 2019). In an extensive study carried out with marine zooplankton, the authors reported two very relevant outputs for the study of MP, which are: organisms ingest aged MP in greater quantity than virgin particles and the ingestion rates are species-specific (Vroom et al. 2017). In this context, the comparison with biological responses after ingestion of other species can serve as a guide, but it is essential that more studies with *C. sancticaroli* are carried out to strengthen the understanding of the potential toxicity of MP for this species.

The ecological relevance of studying aged particles is given by the implications of this process. As changes in the polymeric chemical structure occur, the behavior of MP will tend to be more toxic (Hermabessiere et al. 2017, Zhang et al. 2019). Changes in the chemical structure will promote not only the breaking of chains, but also the adsorption of several other compounds present in the environment, such as organic compounds (Bhagat et al. 2022; Yao et al. 2022). According to the results demonstrated by Luo et al. (2022), changes in the surface of PP after photoaging lead to a greater affinity of this

polymer with organic matter. Thus, in the specific case of chironomids, a point that must be considered is that under environmental conditions, the intake may end up being increased as the particles are also aged, since the feeding of *C. sancticaroli* is not selective. If these particles are more easily ingested, this could also be a pathway for bioaccumulation and biomagnification of MP and absorbed toxic organic compounds through the trophic chain. Thus, MP phototransformation can influence the potential risks of MP to biota through more than one toxicity pathway.

3.3 Mortality of *C. sancticaroli* after exposure period

Mortality data were calculated as cumulative mortality after 144 hours of exposure and expressed as percentage ± SD. After 144 hours, exposure to aged PP-MP reduced the survival of organisms exposed to C1 and C3 concentrations (67.5 and 125 items.g^{-1} of dry sediment, respectively) (Table 2). This effect of decreasing longevity in animals stressed by MP was amplified at the highest concentration (11.6 ± 1.3%).

Table 2
Mortality (% ± SD) of *Chironomus sancticaroli* larvae after 144 hours of exposure under three different concentrations of polypropylene microplastics and negative control (without the presence of the pollutant).

Mortality (%)	
Treatment	After exposure (144h)
Negative control	0.0
C1: 13,5 items.g^{-1}	3.3 ± 1.7
C2: 67,5 items.g^{-1}	0.0
C3: 135 items.g^{-1}	11.6 ± 1.3

Taking into account that mortality was about 3x higher at C3 concentration compared to the mortality presented by organisms treated with C1 concentration, which corresponds to 1/10 of the amount of MP items, this suggests that the concentration interferes with the rate of survival, despite not following a linear curve. The concentration used in the C3 treatment is on the threshold of concentrations found under environmental conditions (He et al. 2020; Klein et al. 2015; Vermaire et al. 2017; Ding et al. 2019), indicating that the mortality of Chironomid larvae may be occurring in environments with these concentrations.

In a study performed with primary PP and a terrestrial invertebrate species, *Metaphire guillelmi*, no mortality was observed during the 14-day exposure time (Cheng et al. 2021). In nauplii and metanauplii of *Artemia salina*, a marine invertebrate, the LC50 was 40.947 µg/mL and 51.954 µg.mL^{-1} and the mortalities were directly proportional to the exposure concentration (Jeyavani et al. 2022). Thus, compared to what was observed in the results of the present work, aged PP-MP may possibly cause greater mortality when interacting with different organisms and, like primary PP, this endpoint is dose-

dependent. In a study with larval culture of acorn barnacle *Amphibalanus amphitrite*, and aged polystyrene MP, mortality was observed to be time-dependent and size-dependent, with 3 µm particles being more toxic than 10 µm MP (Nousheen et al. 2022). Thus, we can expect a greater reduction in survival when the MP are smaller and the concentration and exposure time are greater.

3.4 Biomarkers of oxidative stress

To elucidate the potential effects of PP-MP at the cellular level in *C. sancticaroli* larvae, changes in oxidative stress biomarkers (SOD, CAT, MDA and GST) were investigated. Overall, the investigated biomarkers had a distinct effect dependent on concentration and period of exposure to PP-MP (Fig. 4). While SOD and GST showed no statistically significant changes ($p > 0.05$), CAT and MDA had their levels decreased and increased, respectively, in all treatments compared to the negative control, with the exception of MDA at low concentration, which showed no significant difference.

When the biota is exposed to different adverse conditions, such as the presence of pollutants, free radicals promote reactions with biological substrates, which can cause cellular damage and, consequently, trigger the malfunction of the metabolism as a whole (Samet and Wages 2018). The regulatory system against oxidative stress works to prevent and/or control cellular damage. Each marker plays a role within this system, CAT converts hydrogen peroxide into H_2O and O_2 (Regoli and Giuliani 2014) and MDA is one of the by-products of lipid peroxidation (Esterbauer et al. 1991). Currently, the study of enzymatic alterations has increasingly been applied in research with MP as indicators of cell damage (Han et al. 2022).

In the present study, CAT activity was significantly inhibited in larvae exposed to all concentrations tested (Fig. 4b). MDA levels were significantly increased in larvae exposed to 67.5 and 135 items.g^{-1} of dry sediment (Fig. 4d). These results indicate that exposure to PP-MP for 144 hours caused a deregulation of antioxidant defenses. According to Paul-Pont et al. (2016), CAT may have its activity reduced in response to the ingestion of MP particles by invertebrates. Regarding the increase in MDA activity, the same pattern was observed in *Corbicula fluminea*, a freshwater bivalve, after exposure to polystyrene MP (Fu et al. 2022). In this same study, SOD levels were also increased, which may have occurred due to the longer exposure time (between 7 and 42 days). Still on the results presented here, GST activity was not altered, unlike what was observed in previous studies with *C. riparius* larvae (Silva et al. 2021) exposed to polyethylene MP (~ 40–48 µm) for 48 hours and by other invertebrate species (Avio et al. 2015; Ribeiro et al. 2017). The concentrations used by these studies may also be a relevant factor, as the authors tested considerably higher concentrations, between 1.25 to 20 g of MP per kg^{-1} of sediment. In general, it is possible to notice a lack of consistency between different studies, which may be due to the specificity of each species, the types of MP used and the exposure time.

4 Conclusions

C. sancticaroli organisms were able to ingest PP-MP (26 µm). Mortality was only significant at the highest concentration tested (135 items.g^{-1} of dry sediment). Effects at the cellular level were consistent

for CAT and MDA and, the latter being altered at concentrations 67.5 and 135 items.g^{-1} and CAT also at the lowest concentration, 13.5 items.g^{-1}. It is still unclear whether aged PP-MP can be a threat at the individual or community level, and more extensive and in-depth investigation is recommended with the *C. sancticaroli* species with longer exposure periods and with varying characteristic MP.

Declarations

Credit author statement

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

Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

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References

1. Adler PH, Courtney GW (2019) Ecological and Societal Services of Aquatic Diptera. Insects 10:70. https://doi.org/10.3390/insects10030070.
2. Aebi H (1984) Catalase in vitro. Meth Enzymol 105:121-126. https://doi.org/10.1016/s0076-6879(84)05016-3.
3. Antunes J, Frias JGL, Micaelo AC, Sobral P (2013) Resin pellets from beaches of the Portuguese coast and adsorbed persistent organic pollutants. Estuar Coast Shelf Sci 130:62-69. https://doi.org/10.1016/j.ecss.2013.06.016.
4. Armitage PD, Cranston PS, Pinder LCV (1995) The Chironomidae: Biology and Ecology of Non-Biting Midges. Chapman and Hall 572 p. https://doi.org/10.1007/978-94-011-0715-0.
5. Avio CG, Gorbi S, Milan M, Benedetti M, Fattorini D, d'Errico G, Pauletto M, Bargelloni L, Regoli F (2015) Pollutants bioavailability and toxicological risk from microplastics to marine mussels. Environ Pollut 198:211-222. https://doi.org/10.1016/j.envpol.2014.12.021.

6. Bhagat K, Barrios AC, Rajwade K, Kumar A, Oswald J, Apul O, Perreault F (2022) Aging of microplastics increases their adsorption affinity towards organic contaminants. Chemosphere 298:134238. https://doi.org/10.1016/j.chemosphere.2022.134238.
7. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254. https://doi.org/10.1016/0003-2697(76)90527-3.
8. Campos JF, dos Santos UP, Macorini LFB, Melo AMMF, Balestieri JBP, Paredes-Gamero EJ, Cardoso CAL, Souza KP, Santos EL (2014) Antimicrobial, antioxidant and cytotoxicactivities of propolis from *Melipona orbignyi* (Hymenoptera, Apidae). Food Chem Toxicol 65:374-380. https://doi.org/10.1016/j.fct.2014.01.008.
9. Carpenter EJ, Smith KL (1972) Science 175:1240. https://doi.org/10.1126/science.175.4027.1240.
10. Chen Q, Lackmann C, Wang W, Seiler TB, Hollert H, Shi H (2020) Microplastics lead to hyperactive swimming behaviour in adult zebrafish. Aquat Toxicol 224:105521. https://doi.org/10.1016/j.aquatox.2020.105521.
11. Cheng Y, Song W, Tian H, Zhang K, Li B, Du Z, Zhang W, Wang J, Wang J, Zhu L (2021) The effects of high-density polyethylene and polypropylene microplastics on the soil and earthworm *Metaphire guillelmi* gut microbiota. Chemosphere 267:129219. https://doi.org/10.1016/j.chemosphere.2020.129219.
12. Cincinelli A, Scopetani C, Chelazzi D, Lombardini E, Martellini T, Katsoyiannis A, Fossi MC, Corsolini S (2017) Microplastic in the surface waters of the Ross Sea (Antarctica): occurrence, distribution and characterization by FTIR. Chemosphere 175:391-400. https://doi.org/10.1016/j.chemosphere.2017.02.024.
13. Cummins KW, Klug MJ (1979) Feeding ecology on stream invertebrates. Ann Ver Ecol Syst 10:147-172.
14. Ding L, Mao RF, Guo X, Yang X, Zhang Q, Yang C (2019) Microplastics in surface waters and sediments of the Wei River, in the northwest of China. Sci Total Environ 667:427-434. https://doi.org/10.1016/j.scitotenv.2019.02.332.
15. Doyle D, Sundh H, Almroth BC (2022) Microplastic exposure in aquatic invertebrates can cause significant negative effects compared to natural particles - A meta-analysis. Environ Poll 120434. https://doi.org/10.1016/j.envpol.2022.120434.
16. Eriksen M, Lebreton LCM, Carson HS, Thiel M, Moore CJ, Borerro JC, Galgani F, Ryan PG, Reisser J (2014) Plastic pollution in the World's oceans: more than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. PLoS One 9:e111913. https://doi.org/10.1371/journal.pone.0111913.
17. Esterbauer H, Schaur RJ, Zollner H (1991) Free Radical Biol Med 11:81.
18. Fu L, Xi M, Nicholaus R, Wang Z, Wang X, Kong F, Yu Z (2022) Behaviors and biochemical responses of macroinvertebrate *Corbicula fluminea* to polystyrene microplastic. Sci Total Environ 813:152617. https://doi.org/10.1016/j.scitotenv.2021.152617.

19. Geyer R, Jambeckand JR, Law KL (2017) Production, use, and fate of all plastics ever made. Sci Adv 3:e1700782. http://doi.org/10.1126/sciadv.1700782.
20. Godoy V, Martín-Lara MA, Calero M, Blázquez G (2019) Physical-chemical characterization of microplastics present in some exfoliating products from Spain. Mar Pollut Bull 139:91-99. https://doi.org/10.1016/j.marpolbul.2018.12.026.
21. Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130-7139.
22. Han Y, Zhang X, Liu P, Xu S, Chen D, Liu JN, Xie W (2022) Microplastics exposure causes oxidative stress and microbiota dysbiosis in planarian *Dugesia japonica*. Environ Sci Pollut Res 29:28973-28983. https://doi.org/10.1007/s11356-022-18547-x.
23. Hanvey JS, Lewis PJ, Lavers JL, Crosbie ND, Pozo K, Clarke BO (2017) A review of analytical techniques for quantifying microplastics in sediments. Anal Methods 9:1369-1383. https://doi.org/10.1039/c6ay02707e.
24. He B, Goonetilleke A, Ayoko GA, Rintoul L 2020. Abundance, distribution patterns, and identification of microplastics in Brisbane River sediments, Australia. Sci Total Environ 700:134467. https://doi.org/10.1016/j.scitotenv.2019.134467.
25. Hermabessiere L, Dehaut A, Paul-Pont I, Lacroix C, Jezequel R, Soudant P, Duflos G 2017. Occurrence and effects of plastic additives on marine environments and organisms: a review. Chemosphere 182:781-793. https://doi.org/10.1016/j.chemosphere.2017.05.096.
26. Horton AA, Dixon SJ (2017) Microplastics: an introduction to environmental transport processes. Wiley Interdiscip Rev Water 5:e1268. https://doi.org/10.1002/wat2.1268.
27. Ibáñez-Bernal S, Rivera-García KD, Abella-Medrano CA (2020) Introduction to the taxonomy and general biology of diptera (insecta) involved in the transmission of avian haemosporida. In: Santiago-Alarcon D, Marzal A (eds) Avian Malaria and Related Parasites in the Tropics. Springer, Cham. https://doi.org/10.1007/978-3-030-51633-8_5.
28. Jeyavani J, Sibiya A, Bhavaniramya S, Mahboob S, Al-Ghanim KA, Nisa Z, Riaz MN, Nicoletti M, Govindarajan M, Vaseeharan B 2022. Toxicity evaluation of polypropylene microplastic on marine microcrustacean *Artemia salina*: An analysis of implications and vulnerability. Chemosphere 296:133990. https://doi.org/10.1016/j.chemosphere.2022.133990.
29. Klein S, Worch E, Knepper TP (2015) Occurrence and spatial distribution of microplastics in river shore sediments of the Rhine-Main area in Germany. Environ Sci Technol 49:6070-6076. https://doi.org/10.1021/acs.est.5b00492.
30. Liu L, Fokkink R, Koelmans AA (2016) Sorption of polycyclic aromatic hydrocarbons to polystyrene nanoplastic. Environ Toxicol Chem 35:1650-1655. https://doi.org/10.1002/etc.3311.
31. Luo H, Liu C, He D, Sun J, Zhang A, Li J, Pan X (2022) Interactions between polypropylene microplastics (PP-MPs) and humic acid influenced by aging of MPs. Water Res 222:118921. https://doi.org/10.1016/j.watres.2022.118921.

32. Luo W, Su L, Craig NJ, Du F, Wu C, Shi H (2019) Comparison of microplastic pollution in different water bodies from urban creeks to coastal waters. Environ Pollut 246:174-182. https://doi.org/10.1016/j.envpol.2018.11.081.
33. Müller A, Becker R, Dorgerloh U, Simon F-G, Braun U (2018) The effect of polymer aging on the uptake of fuel aromatics and ethers by microplastics. Environ Poll 240:639-646. https://doi.org/10.1016/j.envpol.2018.04.127.
34. Nousheen R, Rittschof D, Hashmi I (2022) Toxic effects of pristine and aged polystyrene microplastics on selective and continuous larval culture of acorn barnacle *Amphibalanus amphitrite*. Environ Toxicol Pharmacol 94:103912. https://doi.org/10.1016/j.etap.2022.103912.
35. Nel HA, Dalu T, Wasserman RJ (2018) Sinks and sources: assessing microplastic abundance in river sediment and deposit feeders in an Austral temperate urban river system. Sci Total Environ 612:950-956. https://doi.org/10.1016/j.scitotenv.2017.08.298.
36. OECD (2011) Guideline for the testing of chemicals *Chironomus* sp., Acute Immobilisation Test pp 17.
37. OECD/OCDE (2010) Sediment-water Chironomid life-cycle toxicity test using spiked water or spiked sediment. OECD Guidelines for the Testing of Chemicals 233:1-29.
38. Palacio-Cortés AM, Horton AA, Newbold L, Spurgeon D, Lahive E, Pereira MG, Grassi MT, Moura MO, Disner GR, Cestari MM, Gweon HS, Navarro-Silva MA 2022. Accumulation of nylon microplastics and polybrominated diphenyl ethers and effects on gut microbial community of *Chironomus sancticaroli*. Sci Total Environ 832:155089. https://doi.org/10.1016/j.scitotenv.2022.155089.
39. Pinto TJS, Rocha GS, Moreira RA, Silva LCM, Yoshii MPC, Goulart BV, Montagner CC, Daam MA, Espindola ELG 2021. Multi-generational exposure to fipronil, 2,4-D, and their mixtures in *Chironomus sancticaroli*: Biochemical, individual, and population endpoints. Environ Pollut 283:117384. https://doi.org/10.1016/j.envpol.2021.117384.
40. Plastics Europe (2021) Plastics – The Facts 2021. An Analysis of European Latest Plastics Production, Demand and Waste Data. Available at https://plasticseurope.org/knowledge-hub/plastics-the-facts-2021/.
41. Prata JC, Reis V, Matos JTV, Costa JP, Duarte AC, Rocha-Santos T (2019) A new approach for routine quantification of microplastics using Nile Red and automated software (MP-VAT). Sci Total Environ 690:1277-1283. https://doi.org/10.1016/j.scitotenv.2019.07.060.
42. Rebechi D, Palacio-Cortés AM, Richardi VS, Beltrão T, Vicentini M, Grassi MT, Silva SB, Alessandre T, Hasenbein S, Connon R, Navarro-Silva MA 2021. Molecular and biochemical evaluation of effects of malathion, phenanthrene and cadmium on *Chironomus sancticaroli* (Diptera: Chironomidae) larvae. Ecotoxicol Environ Saf 211:111953. https://doi.org/10.1016/j.ecoenv.2021.111953.
43. Regoli F, Giuliani ME (2014) Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Mar Environ Res 93:106-117. https://doi.org/10.1016/j.marenvres.2013.07.006.

44. Ribeiro F, Garcia AR, Pereira BP, Fonseca M, Mestre NC, Fonseca TG, Ilharco LM, Bebianno MJ (2017) Microplastics effects in *Scrobicularia plana*. Mar Pollut Bull 122:379-391. https://doi.org/10.1016/j.marpolbul.2017.06.078.
45. Romano RSG, Oliani WL, Parra DF, Lugao AB (2017) Effects of environmental aging in polypropylene obtained by injection molding. AIP Conf Proc 1914:140001. https://doi.org/10.1063/1.5016766.
46. Rosa BJFV, Rodrigues LFT, Oliveira GS, Alves RG (2014) Chironomidae and Oligochaeta for water quality evaluation in an urban river in southeastern Brazil. Environ Monit Assess 186:7771-7779. https://doi.org/10.1007/s10661-014-3965-5.
47. Samet JM, Wages PA (2018) Oxidative stress from environmental exposures. Curr Opin Toxicol 7:60-66. PMID: 30079382.
48. Scherer C, Brennholt N, Reifferscheid G, Wagner M (2017) Feeding type and development drive the ingestion of microplastics by freshwater invertebrates. Sci Rep 7:1-9. https://doi.org/10.1038/s41598-017-17191-7.
49. Scherer C, Wolf R, Völker J, Stock F, Brennhold N, Reifferscheid G, Wagner M 2019. Toxicity of microplastics and natural particles in the freshwater dipteran *Chironomus riparius*: same same but different? Sci Total Environ 711:134604. https://doi.org/10.1016/j.scitotenv.2019.134604.
50. Serra SRQ, Graça MAS, Dolédec S, Feio MJ (2017) Chironomidae traits and life history strategies as indicators of anthropogenic disturbance Environ Monit Assess 189:326. https://doi.org/10.1007/s10661-017-6027-y.
51. Silva CJM, Patrício AL, Gravato C, Pestana JLT (2019) Ingestion of small-sized and irregularly shaped polyethylene microplastics affect *Chironomus riparius* life-history traits. Sci Total Environ 672:862-868. https://doi.org/10.1016/j.scitotenv.2019.04.017.
52. Silva CJM, Silva ALP, Campos D, Machado AL, Pestana JLT, Gravato C (2021) Oxidative damage and decreased aerobic energy production due to ingestion of polyethylene microplastics by *Chironomus riparius* (Diptera) larvae. J Hazard Mat 402:123775. https://doi.org/10.1016/j.jhazmat.2020.123775.
53. Stock F, Kochleus C, Bäansch-Baltruschat B, Brennholt N, Reifferscheid G (2019) Sampling techniques and preparation methods for microplastic analyses in the aquatic environment – a review. Trends Anal Chem 113:84-92. https://doi.org/10.1016/j.trac.2019.01.014.
54. Strixino ST, Strixino G (1982) Ciclo de vida de *Chironomus sancticaroli* Strixino & Strixino, (Diptera, Chironomidae). Rev Bras Entomol 26:183-189.
55. Torres FG, Dioses-Salinas DC, Pizarro-Ortega CI, De-la-Torre GE (2020) Sorption of chemical contaminants on degradable and non-degradable microplastics: recent progress and research trends. Sci Total Environ 757:143875. https://doi.org/10.1016/j.scitotenv.2020.143875.
56. Vermaire JC, Pomeroy C, Herczegh SM, Haggart O, Murphy M (2017) Microplastic abundance and distribution in the open water and sediment of the Ottawa River, Canada, and its tributaries. Facets 2:301-314. https://doi.org/10.1139/facets-2016-0070.
57. Vroom RJE, Koelmans AA, Besseling E, Halsband C (2017) Aging of microplastics promotes their ingestion by marine zooplankton. Environ Poll 231:987-996.

https://doi.org/10.1016/j.envpol.2017.08.088.

58. Wagner M, Lambert S (2017) Freshwater Microplastics. Emerging Environmental Contaminants? The Handbook of Environmental Chemistry 58. Ed. Springer: Cham 303 p.
59. Wan Z, Wang C, Zhou J, Shen M, Wang X, Fu Z, Jin Y (2019) Effects of polystyrene microplastics on the composition of the microbiome and metabolism in larval zebrafish. Chemosphere 217:646-658. https://doi.org/10.1016/j.chemosphere.2018.11.070.
60. Yao J, Wen J, Li H, Yang Y (2022) Surface functional groups determine adsorption of pharmaceuticals and personal care products on polypropylene microplastics. J Hazard Mat 423:127131. https://doi.org/10.1016/j.jhazmat.2021.127131.
61. Zhang C, Zhou H, Cui Y, Wang C, Li Y, Zhang D (2019). Microplastics in offshore sediment in the Yellow Sea and East China Sea, China. Environ Pollut 244:827-833. https://doi.org/10.1016/j.envpol.2018.10.102.
62. Ziajahromi S, Kumar A, Neale PA, Leusch FDL (2018) Environmentally relevant concentrations of polyethylene microplastics negatively impact the survival, growth and emergence of sediment-dwelling invertebrates. Environ Pollut 236:425-431. https://doi.org/10.1016/j.envpol.2018.01.094.

Figures

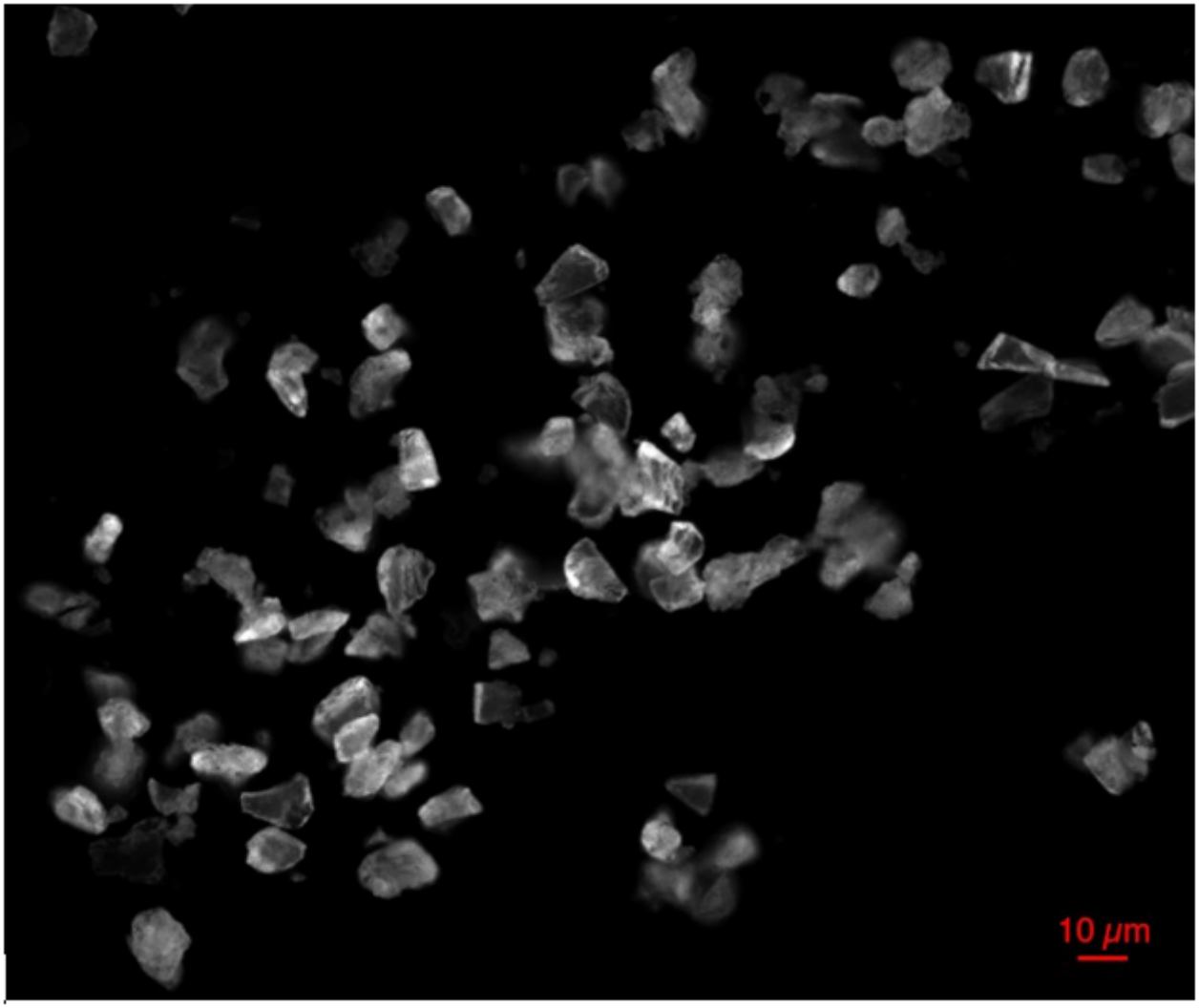

Figure 1

Visualization of aged PP-MP particles stained with Nile red under inverted fluorescence microscopy.

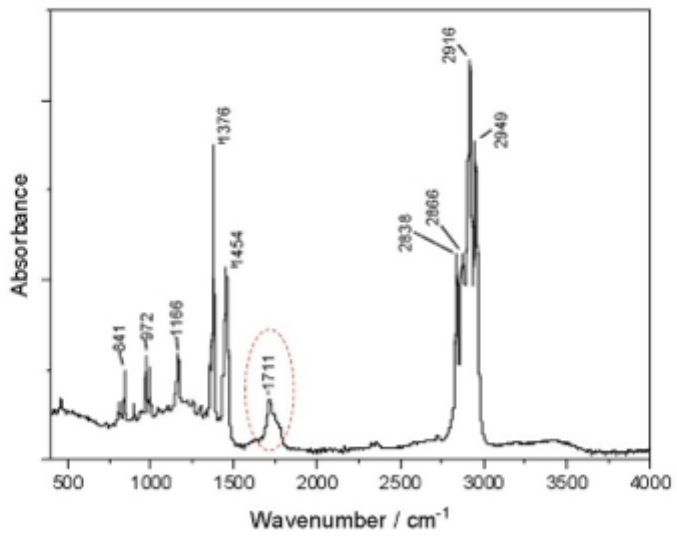

Figure 2

Characterization of the microplastic sample used in the experiment by Fourier transform spectroscopy (FTIR) confirming the polymer type as aged polypropylene (PP). The peak indicating the presence of carbonyl groups due to aging is highlighted with a red dotted circle at 1711 cm^{-1}.

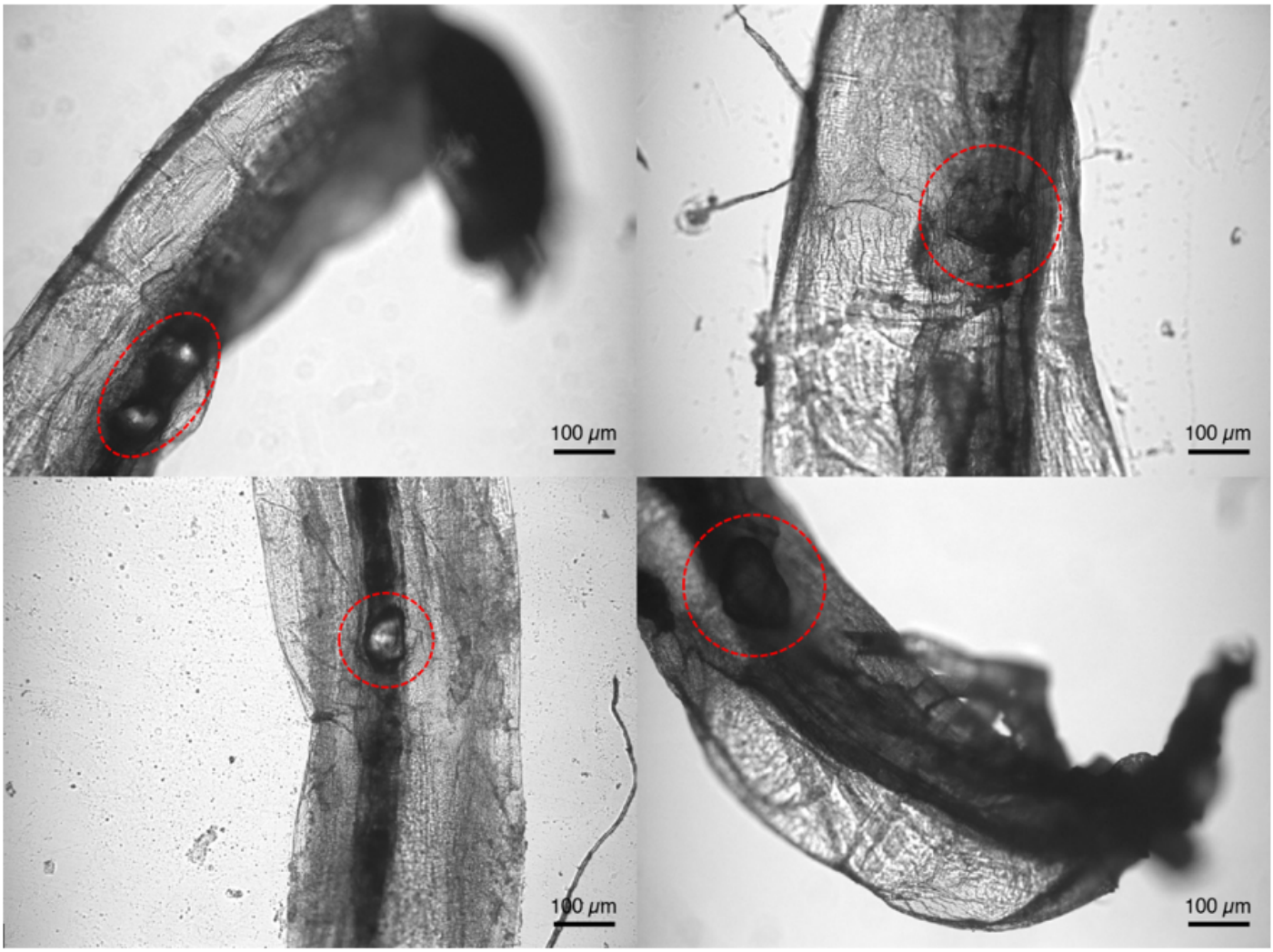

Figure 3

Representative sample of ingestion of naturally aged polypropylene microplastics (PP-MP) by *Chironomus sancticaroli* during the exposure period (144 hours) at three different concentrations (13.5; 67.5 and 135 items.g^{-1} of dry sediment).

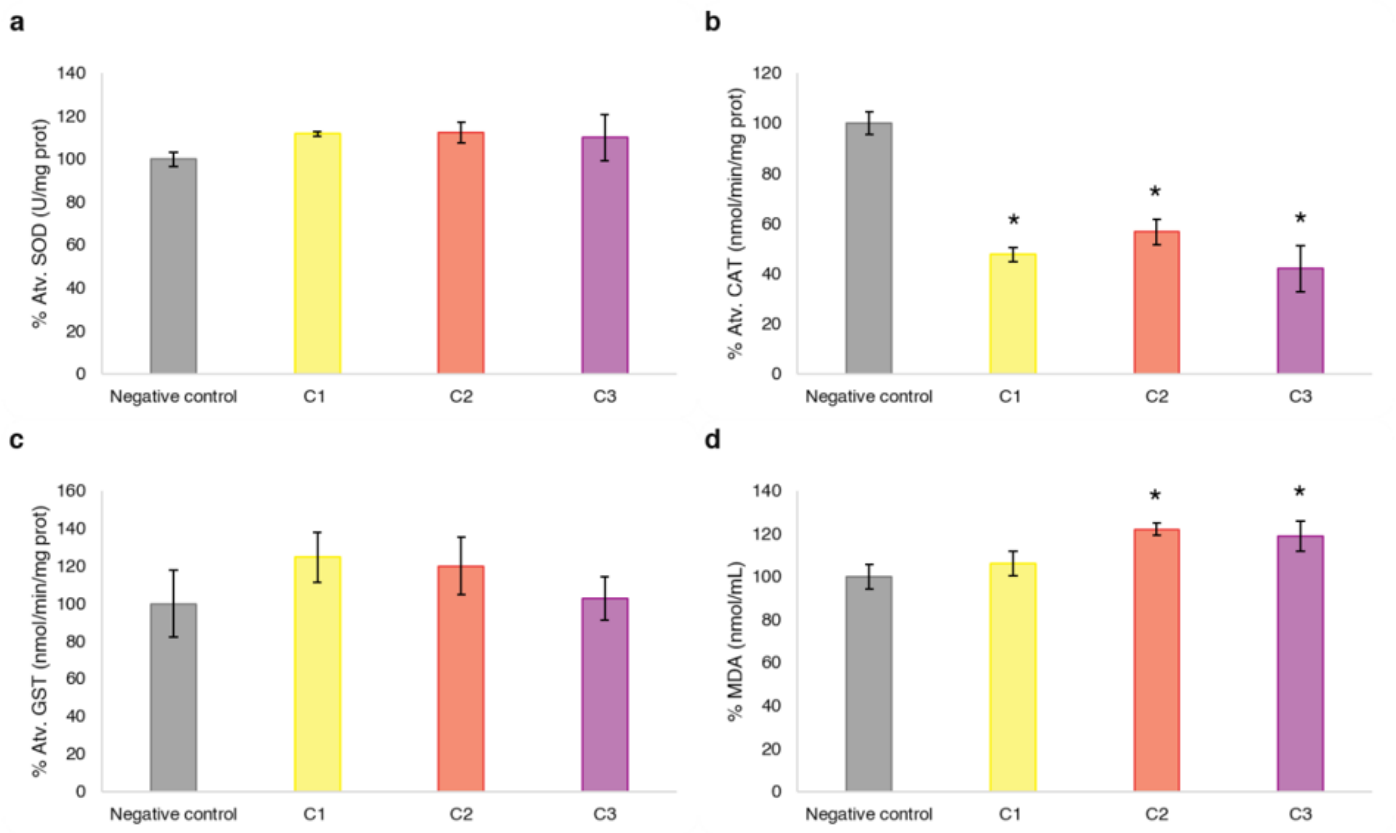

Figure 4

Changes in oxidative stress markers in response to 144 hours of exposure to polypropylene microplastics (PP-MP) at three different concentrations (C1: 13.5, C2: 67.5, C3: 135 items.g^{-1} of dry sediment). The levels of (a) SOD, (b) CAT, (c) GST and (d) MDA in *Chironomus sancticaroli* are shown according to protein content. The asterisk symbol (*) represents a statistically significant difference ($p < 0.05$) in oxidative stress markers between the treatment and the negative control.