Journal Pre-proof

Oxidative damage and decreased aerobic energy production due to ingestion of polyethylene microplastics by *Chironomus riparius* (Diptera) larvae

Carlos JM Silva (Conceptualization) (Investigation)<ce:contributor-role>Data acquisition) (Formal analysis)<ce:contributor-role>Writing-original draft)<ce:contributor-role>Writing-review and editing), Ana L. Patrício Silva (Funding acquisition) (Project administration)<ce:contributor-role>Data acquisition) (Formal analysis)<ce:contributor-role>Writing-review and editing), Diana Campos (Investigation)<ce:contributor-role>Data acquisition) (Formal analysis)<ce:contributor-role>Writing-review and editing), Ana L. Machado<ce:contributor-role>Writing-review and editing), João LT Pestana (Conceptualization) (Supervision)<ce:contributor-role>Writing-review and editing), Carlos Gravato (Conceptualization) (Supervision)<ce:contributor-role>Writing-review and editing)

PII: S0304-3894(20)31764-7

DOI: https://doi.org/10.1016/j.jhazmat.2020.123775

Reference: HAZMAT 123775

To appear in: *Journal of Hazardous Materials*

Received Date: 17 June 2020

Revised Date: 6 August 2020

Accepted Date: 20 August 2020

Please cite this article as: Silva CJ, Silva ALP, Campos D, Machado AL, Pestana JL, Gravato C, Oxidative damage and decreased aerobic energy production due to ingestion of polyethylene microplastics by *Chironomus riparius* (Diptera) larvae, *Journal of Hazardous*

Materials (2020), doi: https://doi.org/10.1016/j.jhazmat.2020.123775

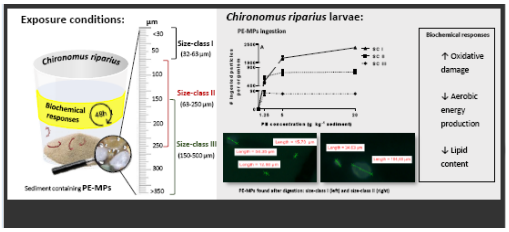
Oxidative damage and decreased aerobic energy production due to ingestion of polyethylene microplastics by *Chironomus riparius* (Diptera) larvae

Carlos JM Silva[1*], Ana L. Patrício Silva[1], Diana Campos[1], Ana L. Machado[1], João LT Pestana[1], Carlos Gravato[2]

[1]CESAM - Centre for Environmental and Marine Studies, Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[2]Faculty of Sciences & CESAM, University of Lisbon, Campo Grande 1749-016 Lisbon, Portugal

Graphical Abstract



Highlights

- *C. riparius* larvae ingested PE-MPs from contaminated sediment after 48 hours
- Accumulation of 32-63 µm size range particles in larvae guts was highest
- Ingestion of MPs induced oxidative damage and reduced aerobic energy production
- Depletion of lipid content were also observed in larvae under exposure to PE-MPs

Abstract

Riverine sediments are major sinks of microplastics from inland anthropogenic activities, imposing a threat to freshwater benthic invertebrates.

This study investigated the ingestion of three size-classes (SC) of irregularly shaped polyethylene microplastics (PE-MPs; SC I: 32-63 µm; II: 63-250 µm; III: 125-500 µm) after 48 hours by dipteran larvae (detritivore/collector) *Chironomus riparius,* and the consequent effects on neurotransmission, energy allocation and oxidative stress. The tested PE-MPs concentrations (1.25; 5; 20 g kg^{-1}) were within the range of concentrations reported in riverbanks from highly urbanised areas (1 - 9 g kg^{-1}), except for 20 g kg^{-1} representing the worst-case scenario. After exposure to SC I, larvae presented high amounts (up to ~2400 particles/larvae) of PE-MPs in their guts, with an average size-range of 30-60 µm. In the SC II and III, larvae presented PE-MPs of higher diameter (up to 125 µm) and a visible gut obstruction. The high number of particles in the larval gut (SC I) and/or difficulties for their egestion (SC I, II and III) induced oxidative damage and reduced aerobic energy production. In addition, larvae exposed to SC II and III revealed depletion in their total lipid reserves as a consequence of lacking nutrients, and the ones exposed to SC III presented a decrease in their detoxification capacity.

These results highlight that freshwater detritivores with low selective feeding behaviour (e.g., chironomids) are more prone to ingest microplastics, with potentially adverse effects on cellular metabolism, redox status and antioxidant-detoxification defences. These harmful effects at lower levels of the biological organisation may ultimately affect organisms' physiology and fitness.

Keywords: Benthic Invertebrates; Energy Reserves; Biomarkers; Aquatic insects; Acute Effects

1. Introduction

Microplastics (MPs, synthetic or semi-synthetic polymer with size between 1 µm to 5 mm; Frias and Nash, 2019) reach freshwater ecosystems in its microscale (primary MPs) or as a result of degradation of larger pieces of plastic (secondary MPs). Once in freshwaters, MPs undergo biological and physical processes such as biofouling and homo- or hetero-aggregation (Lagarde et al., 2016; Lambert et al., 2017) that are known to modify MPs' density and floatability and to accelerate its deposition in sediments (Corcoran, 2015). Continuous release and long-term deposition make MPs present and persistent in freshwater sediments, especially near highly industrialised or densely populated areas (Klein et al., 2015; Castañeda et al., 2014) where they can reach levels up to 9 g kg^{-1}, as in Mersey/Irwell river, United Kingdom (Hurley et al., 2018).

The contamination of freshwater sediments by MPs is of special concern, as many key-benthic invertebrates (on nutrient cycling) feed on the particulate organic matter within the same size-range of most primary MPs (Henriques-Oliveira et al., 2003; Syrovátka et al., 2009; Conkle et al., 2017). The ingestion of MPs has been related to possible harmful developmental and reproductive effects on several freshwater benthic invertebrate species, such as *Chironomus tepperi* (Ziajahromi et al., 2018), *Chironomus riparius* (Scherer et al., 2019; Silva et al., 2019, Stanković et al., 2020; Khosrovyan et al., 2020), and *Hyalella azteca* (Au et al., 2015). Thus, the contamination of freshwater sediments by MPs is of

special concern, since nutrient cycling would be impaired if key-benthic invertebrates feed on MPs instead of particulate organic. Also, the presence of MPs in freshwater sediments seems to alter benthic communities (Redondo-Hasselerharm et al., 2020). In contrast, other recent studies found limited to no effects on freshwater invertebrates' life-history traits (Weber et al., 2018; Khosrovyan and Kahru, 2020), but the studies were performed using different polymers (polyamide and polyethylene terephthalate) and did not address ingestion/egestion capacities. The effects of MPs at the cellular and sub-cellular levels on freshwater invertebrates are less studied and can represent early warning signals of sub-lethal effects providing valuable information concerning the mechanisms of action of MPs (Jeong and Choi, 2019). Despite this lack of information for freshwater biota, there are already some insights on biochemical responses triggered after exposure to MPs (González-Pleiter et al., 2019); but were mainly performed in filter-feeding organisms and most studies only report the ingestion of MPs, but do not quantify the particles present inside the gut of the organisms. Relevant physiological and biochemical responses induced by microplastics include (1) energy impairment (Wright et al., 2013a; Gardon et al., 2018); (2) neurotoxicity (Oliveira et al., 2013; Luís et al., 2015); (3) activation of immune system responses (Avio et al., 2015; Veneman et al., 2017); (4) oxidative stress (Jeong et al., 2016; Lu et al., 2016; Chen et al., 2017; Deng et al., 2017; Choi et al., 2018; Espinosa et al., 2018); and (5) changes in the microbiome (Jin et al., 2018; Lu et al., 2018).

Recognising that long-term ingestion of polyethylene microplastics (PE-MPs) cause significant alterations in life-history traits in *C. riparius* (Silva et al., 2019) and that polyethylene is one of the most common polymers found in freshwater sediments (Li et al., 2019), this study aimed to determine the number and size of ingested particles after short-term exposure to contaminated sediments and to assess the alterations induced on key-physiological functions of *C. riparius* larvae that are triggered by the particles retained in the gut. For that, fourth instar *C. riparius* larvae were exposed for 48 hours to three different concentrations (1.25-5-20 g kg^{-1}) of PE-MPs of three different size-classes (I:32–63; II:63–250 and III:125-500 µm). The range of concentrations used for testing was set based on levels reported for freshwater sediments in highly urbanised areas (Hurley et al., 2018). Physiological responses were evaluated using biomarkers to assess the health condition of larvae including oxidative damage (lipid peroxidation, LPO), phase II biotransformation enzyme (glutathione-*S*-transferase, GST), enzymatic antioxidant defences (catalase, CAT), an enzymatic activity important for cholinergic neurotransmission (acetylcholinesterase, AChE), energy reserves (lipids, proteins and sugars), and electron transport system (ETS) activity (aerobic energy production).

2. Materials and methods

2.1 Organism culture conditions

Chironomus riparius larvae that were used in the experiment were reared at the University of Aveiro (Department of Biology). Briefly, *C. riparius* are

maintained at 20 ± 1°C under a 16:8 h light-dark photoperiod. Larvae are grown in glass aquaria containing a layer (~3cm) of previously burnt (500 °C for 4h) inorganic sediment (<1 mm) and American Society for Testing Materials (ASTM) hard water in a 1:4 ratio (ASTM, 1980), with continuum aeration. Glass aquaria used for rearing *C. riparius* larvae are confined within acrylic cage retaining the adults. *C. riparius* culture is fed (*ad libitum*) three times a week using a suspension of macerated TetraMin® (Tetrawerke, Melle, Germany). The ASTM hard water is biweekly renewed, and sediment is monthly replaced. *C. riparius* life cycle is finalised within 3-4 weeks in laboratory-controlled conditions.

Fourth instar larvae (12 days old post-hatching) from egg ropes isolated from the culture were used in the bioassays.

2.2 Polyethylene microplastics used in the experiment

Polyethylene (PE) microplastics of different size were purchased to be used in the experiments. The technical information on the PE-MPs used in the tests (size-classes I, II and III) and respective particle size distribution after vibratory sieve shaking is presented in table 1. The particle size presented corresponds to ≥ 90% of the particles present in the respective size-class.

Table 1: Technical information on the polyethylene microplastics used for testing.

Size-class	Average size	≥ 90% of particles size	CAS	Company

I	40-48 µm	32-63 µm	9002-88-4	Sigma-Aldrich UK
II	125 µm	63-250 µm	9002-88-4	Sigma-Aldrich UK
III	350 µm	125-500 µm	708-316-83	Goodfellow USA

Polyethylene microplastics size-range was the same as previous tests (Silva et al., 2019), which is within the size of regular food items ingested by the *C. riparius* larvae (Epler, 1995; Henriques-Oliveira et al., 2003; Ristola et al., 1999).

Airborne plastic contamination was prevented by using glassware as a replacement for plasticware whenever possible. Thereby, technical controls were also adjoined to discount any possible airborne MP contamination during extraction and quantification procedures.

2.3 Short-time exposure (48 h) to PE-MPs contaminated sediment

For biochemical biomarkers and cellular energy allocation (energy reserves and aerobic energy production) measurements, fourth instar (12-day old) *C. riparius* larvae were exposed to a gradient of MP concentrations (1.25; 5 and 20 g kg^{-1} dry sediment) of PE-MPs of three different size-classes (I: 32-63 µm, II: 63-250 µm and III: 125-500 µm). It is important to note that these concentrations were chosen to include levels reported in the field (Hurley et al., 2018) and concentrations that are known to induce deleterious effects on *C. riparius* growth and emergence (Silva et al., 2019). Each condition plus a control treatment (uncontaminated inorganic sediment) demanded seven replicates, containing

fifteen larvae each. Larvae were exposed in glass vials containing PE-MPs mixed (except for control treatment) in 50 g of fine sediment (<1mm) and 150 mL of ASTM hard water. No food was provided during the experiment. After 48 h, larvae were washed using ultra-pure water to remove potential microplastic particles adhered to the larvae and quickly dried on filter paper. After collection, the larvae were immediately weighed, frozen in liquid nitrogen, and stored at –80 °C until use. Three additional replicates were used to estimate the MP ingestion by *C. riparius* larvae after the 48 hours exposure period.

2.4 Biochemical biomarkers assessment in *C. riparius* larvae

All biochemical determination followed optimised protocols for a microplate reader (Campos et al., 2016; Rodrigues et al., 2014; 2015). Briefly, biological samples were homogenised (Qiagen TissueLyser II) for 30 seconds at 4 °C in 1600 µL of Milli-Q water in 2 mL microtubes. Two aliquots of 300 µL were collected from each sample for the analysis of energy reserves: one aliquot was used for the analysis of lipids content, and the other aliquot was used for the determination of sugars and proteins content. From the remaining homogenate, 300 µL aliquot was collected to estimate aerobic energy production - measured by evaluating the electron transport system (ETS) activity; and 200 µL aliquot was used for the determination of lipid peroxidation (LPO). The remaining volume of the homogenate (~500 µL) was diluted in 500 µL of 0.2M K-phosphate buffer, pH 7.4. The resulting solution was centrifuged at 9,000 g (4 °C) for 20 min, and the post-mitochondrial supernatant (PMS) split into microtubes for subsequent

enzymatic determination. The resulting PMS samples were stored at −80 °C until enzymatic activity determination of catalase (CAT), glutathione-*S*-transferase (GST), acetylcholinesterase (AChE) activities, and also the quantification of protein levels in the samples.

The energy reserves (sugar, lipid, and protein contents), representative of the energy available as well as the aerobic energy production (ETS activity) were assessed following De Coen and Janssen (Coen and Janssen, 1997). As for the total lipid content, aliquots were centrifuged after addition of chloroform (500 µL) and methanol (500 µL). After centrifugation, the organic phase of each sample and tripalmitin standard solution were moved to glass tubes and acidified using sulfuric acid (H_2SO_4; 500 µL). The absorbance was determined at 375 nm on the microplate reader.

Protein content was estimated after precipitation by 15% trichloroacetic acid, followed by incubation at –20 °C. Samples were then centrifuged (1000 g for 10 min at 4 °C), and the supernatant was separated to be used in sugar measurement. For sugar content estimation, samples and glucose standard concentrations were incubated after adding 5% phenol and H_2SO_4. The absorbance was then read at 492 nm to quantify sugar content. In the same sample, the pellets were resuspended after sodium hydroxide (NaOH), incubated (60 °C for 30 min), and pH was neutralised using HCl. Total protein content was quantified by Bradford's method in which the absorbance was read at 592 nm and using bovine serum albumin for the standard concentration curve (Bradford, 1976).

The aliquot of the homogenate used for ETS activity measurements (300 µL) was treated by adding Tris base homogenisation buffer (0.3 M), polyvinylpyrrolidone (0.45% (w/v), $MgSO_4$ (459 µM), Triton X-100 ph 8.5 (0.6% (v/v), and centrifuged (1000 g for 10 min at 4 °C). Before measurements, 50 µL of the resulting supernatant was incubated with a buffered solution (0.13 M Tris base with 0.27% [v/v] Triton X-100, 1.7 mM reduced nicotinamide adenine dinucleotide, 274 µM reduced nicotinamide adenine dinucleotide phosphate, and INT (p-iodonitrotetrazolium; 8 mM solution). The oxygen consumption was estimated by following the kinetics of the absorbance at 490 nm throughout a 3-min period.

Lipid peroxidation was verified by assessing thiobarbituric acid-reactive substances (TBARS) at 535 nm (Bird and Draper, 1984), GST activity determination was performed by measuring the colorimetric reaction (340 nm) of GSH conjugation with 1-chloro-2,4-dinitrobenzene (Habig et al., 1974), and CAT activity was determined by measuring the colorimetric reaction of the H_2O_2 decomposition at 240 nm (Claiborne, 1985). Acetylcholinesterase activity was determined by following the absorbance at 412 nm using acetylthiocholine as substrate according to Ellman's method (Ellman et al., 1961). The protein levels in each sample were determined following the Bradford method, adapted from BioRad's Bradford microassay set up in a 96 well microplate and using bovine γ-globulin as a standard (Bradford, 1976).

2.5 Extraction and quantification of PE microplastics in *C. riparius* larvae

After exposure, five larvae of each replicate were acid digested and further used for the extraction and quantification of MPs inside the gut. Quickly, samples were lyophilised for 24 h and weighed (DW). After dehydration, larvae were placed into glass flasks and cautiously macerated using a small glass rod (acting as a mortar). This procedure allowed biological samples to be easily digested latter. The digestion procedure was comprised after the addition of 3 mL of nitric acid (HNO_3; 65%) and further incubation at 60°C for three hours. After this incubation, samples were cooled down to room temperature (RT), and a volume of 2.6 mL of hydrogen peroxide (H_2O_2; 35%) was added to complete the digestion (Lusher et al., 2017). After 24 h, and once no visible oxygen bubbles were being released (signal of successful digestion), the samples were diluted using Milli-Q water in a 1:10 ratio. The resulting solution was then vacuum filtered onto gridded cellulose ester filters (Whatman 10406972, Mixed Cellulose Ester Filter, 3.1 mm white/black grid, 0.45 µm pore size). Retained particles (in the filtration device) were rinsed using Milli-Q water to minimise estimation errors. Filter membranes containing the microplastics were transferred to glass Petri-dishes and left to dry at 25 °C for 2–3 days. Afterwards, the number of particles present inside the larvae was counted under a stereomicroscope. To calculate the average size of these particles, the major diameter (Fig. S1) of all microplastics located in five sorted squares of each filter was measured. A stereomicroscope (stereoscopic zoom microscope—SMZ 1500, Nikon Corporation) associated with NIS-Elements D 3.2 imaging software were used for the necessary measurements (check supplemental data).

2.6 Statistical analysis

Non-parametric Spearman correlation was used to analyse the relationship between MPs concentration and the number of microplastics inside larval gut for each pool.

Normality and variance homogeneity assumptions were confirmed by analysing the residuals. One-way analysis of variance (ANOVA) was used for each PE-MP size-class to analyse differences between treatments for each size-class. Dunnett's test was used to determine significant differences from control. Statistical differences were considered at $p<0.05$. All statistical analysis was performed using R version 3.6.1 (Core Team R, 2019).

3. Results

3.1 Ingestion of PE-MPs by *Chironomus riparius* larvae after 48 h exposure time

The number of PE-MPs ingested by larvae was higher in size-class (SC) I that contained smaller-size PE-MPs < 63 µm. Thus, larvae exposed to SC I ingested an average of 525, 2047 and 2389 PE-MPs at concentrations of 1.25, 5 and 20 g kg^{-1} sediment, respectively; whereas larvae ingested 656 to 785 PE-MPs in SC II or approximately 75 PE-MPs in SC III, independently of the concentration tested. Moreover, the number of ingested particles was correlated to increasing PE-MPs concentration in sediments for SC I (r= 1.000, p=0.0417) and II (r= 1.000, p=0.0417), but not for SC III (r= 0.400, p=0.3750) (Fig. 1A).

The average size of PE-MPs ingested was higher on larvae exposed to any concentration of the SC II (48.78 ± 1.39 µm) and III (59.69 ± 2.16 µm) than in the ones exposed to SC I (38.76 ± 0.61 µm; Fig. 1B and Supplemental data Fig. S1). However, most of the ingested microplastics on larvae exposed to all SC of PE-MPs was within the 32-63 µm size-range, which in the case of SC I accounted for more than 90% of the total particles, but less than 10% for SC II and III (Table 1).

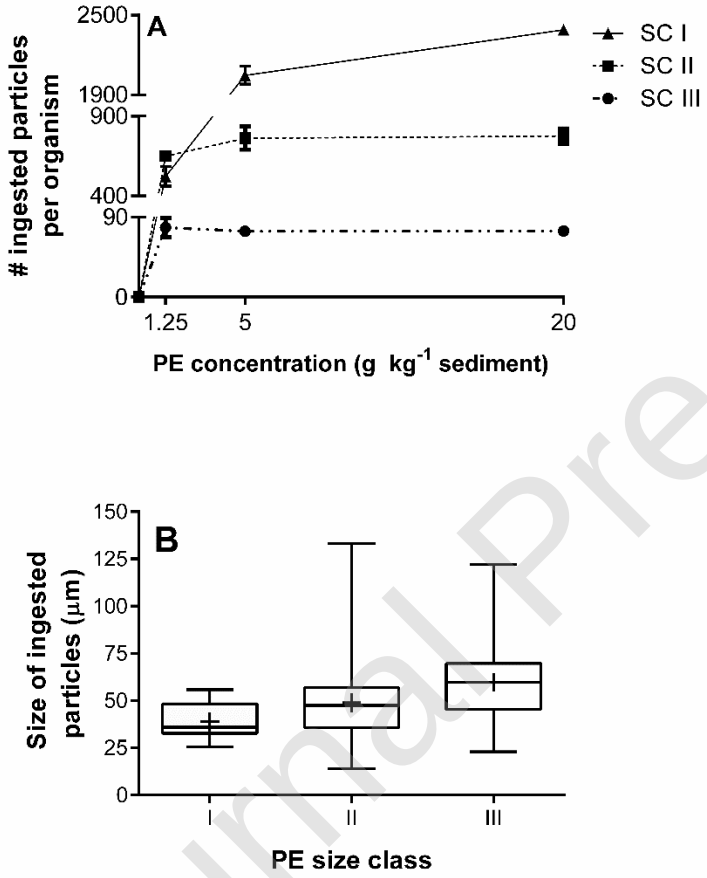


Fig. 1: Number (A) and size (B) of polyethylene microplastics (PE-MPs) of three size-classes (I, II and III) ingested by *Chironomus riparius* larvae, after 48 h of exposure. The number of ingested PE-MPs (A) is displayed as the mean ± standard error of the mean (*n*=3). For some data, the error bars can be imperceptible. The size of the ingested PE-

MPs (B) is presented as mean (± symbol) with interquartile range, whiskers, and maximum/minimum values.

3.2 Energy reserves and aerobic energy production

Exposed larvae showed a decrease in aerobic energy production, regardless of PE-MPs SC (Fig. 2). The ETS activity significantly decreased in larvae exposed to 5 and 20 g kg^{-1} of SC I ($F_{3,24}$=7.810, p<0.001;); all concentrations of SC II ($F_{3,24}$=18.05, p<0.001) and 1.25 and 5 g kg^{-1} of SC III ($F_{3,23}$=5.447, p=0.006;). Lipid content significantly decreased in larvae exposed to 1.25, 5 and 20 g kg^{-1} of SC II ($F_{3,24}$=6.045, p=0.003) and III ($F_{3,24}$=4.677, p=0.011) (Fig. 2). Sugar ($F_{3,23}$=0.8375, p=0.487; $F_{3,24}$=3.989, p=0.019; $F_{3,23}$=1.065, p=0.383 for SC I, II and III respectively) and protein contents ($F_{3,24}$=0.878, p=0.466; $F_{3,24}$=1.755, p=0.183; $F_{3,23}$=0.818, p=0.497; for SC I, II and III respectively) did not vary between larvae exposed to control conditions and larvae exposed to any PE-MP treatments size-class (Fig. 2).

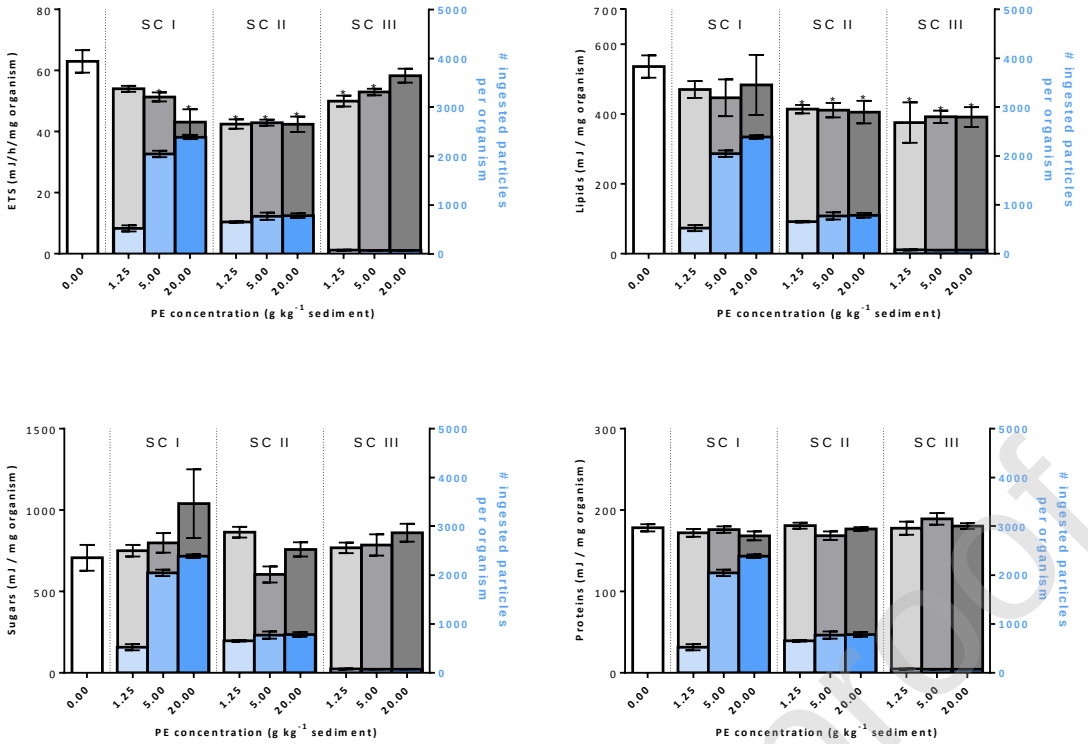


Fig. 2: Effect of short-term exposure (48 h) to polyethylene microplastics (PE-MPs) of three size-classes (I: 32–63 µm, II: 63–250 µm, and III: 125–500 µm, from left to right) on aerobic energy production (electron transport system, ETS, mJ/h/mg organism); lipids, sugars, and protein content (mJ/mg organism) of *Chironomus riparius* 4th instar larvae. All values are presented as mean ± standard error of the mean (*n*=7). *denotes a significant (p< 0.05) difference when compared with the control (0) treatment following ANOVA and post-hoc Dunnett's tests. A gradient of blue bars represents the number of ingested MPs by larvae, as shown in figure 1A. For interpretation of the references to colour in this figure caption, the reader is referred to the web version of this paper.

3.3 Oxidative stress and Detoxification

Lipid peroxidation (LPO) significantly increased (approximately 70 %) on larvae of *C. riparius* exposed to all the concentrations of the SC I ($F_{3,24}$=26.57,

p<0.001), SC II ($F_{3,24}$=18.15, p<0.001) and SC III ($F_{3,22}$=14.72, p<0.001) PE-MPs (Fig. 3).

Catalase activity was significantly inhibited on larvae exposed to all concentrations of SC III ($F_{3,23}$=6.917, p=0.002) and 5 g kg^{-1} of SC I ($F_{3,24}$=4.464, p=0.013; Fig. 3). GST activity was significantly inhibited on larvae exposed to all concentrations of SC III ($F_{3,23}$=4.762, p=0.010; Fig. 3). Acetylcholinesterase activity was significantly increased on larvae exposed to 20 g kg^{-1} of SC III ($F_{3,23}$=6.863, p=0.002; Fig. 3).

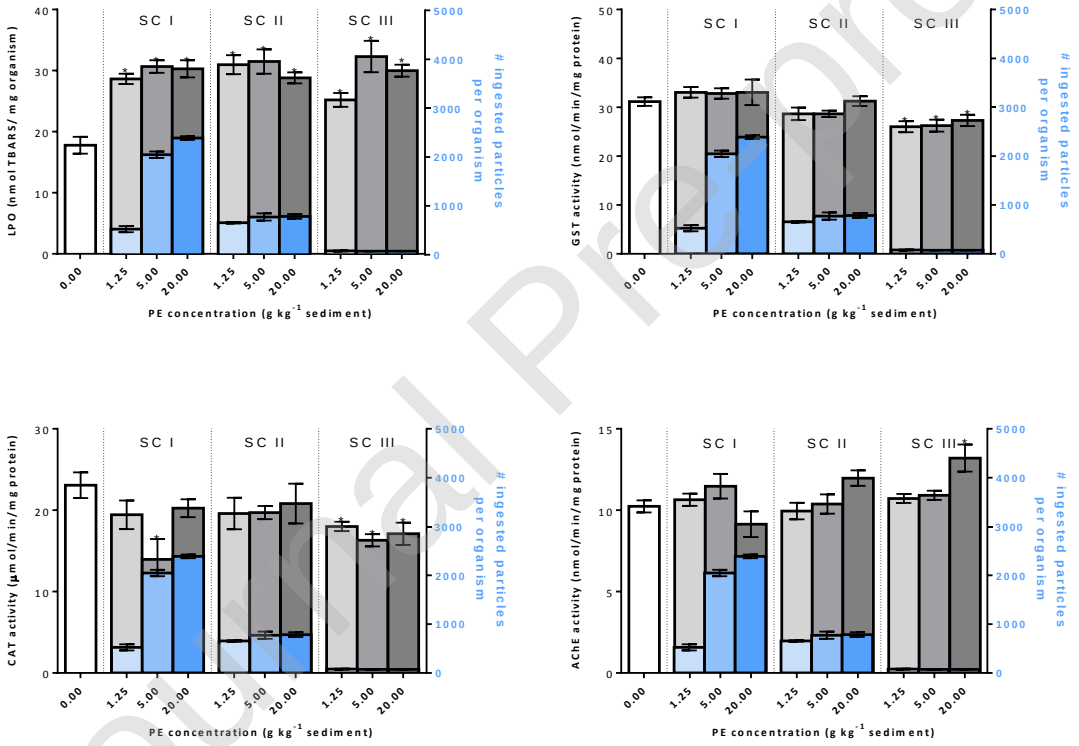


Fig. 3: Effect of short-term exposure (48 h) to polyethylene microplastics (PE-MPs) of three size-classes (I: 32–63 µm, II: 63–250 µm, and III: 125–500 µm, from left to right) on lipid peroxidation (LPO, nmol TBARS/mg protein), catalase activity (CAT,

µmol/min/mg protein), glutathione-*S*-transferase levels (GST, nmol/min/mg protein) and acetylcholinesterase (AChE, nmol/min/mg protein) of *Chironomus riparius* 4th instar larvae. All values are presented as mean ± standard error of the mean (*n*=7). *denotes a significant ($p < 0.05$) difference when compared with the control (0) treatment following ANOVA and post-hoc Dunnett's tests. The gradient of blue bars represents the number of ingested MPs by larvae, as shown in figure 1A. For interpretation of the references to colour in this figure caption, the reader is referred to the web version of this paper.

Discussion

This study provides the first insight on the deleterious effects on cellular energy allocation, energy production, oxidative stress and neurotoxicity induced by the different size and number of microplastics ingested by larvae of the benthic freshwater insect *Chironomus riparius*.

As sediment-dwelling organisms, chironomid' larvae feed on the particulate organic matter without much selectivity (Armitage et al., 1995), which make them one of the most susceptible organisms to the potential ingestion of MPs that sink and accumulate in sediments. The ingestion of considerable amounts of microplastics was already reported in chironomid larva both from field samples (Nel et al., 2018) and from laboratory organisms after short-term (3 h) (Scherer et al., 2017) and long-term (≥ 5 days) (Ziajahromi et al., 2018; Silva et al., 2019) exposures. Given its non-selective feeding behaviour, the ingestion of MPs observed in larvae of *C. riparius* seems to be within the same range of the size of particles naturally ingested (up to 60 µm), and its accumulation in the gut is mostly dependent on organisms feeding apparatus and their egesting capabilities.

The number of PE-MPs inside *C. riparius* larval gut observed after 48 h agrees with our previous investigation (Silva et al., 2019). However, in such previous investigation, *C. riparius* larvae were exposed to PE-MP treatments (same concentrations and size-class) from their 1st instar and up to 10 days (so larvae were at their 4th instar after exposure period). The comparison of ingestion data and time of exposure from both studies suggests that: i) larvae ingest higher quantities of PE-MP particles in their 4th instar; ii) larvae may regularly be ingesting and egesting PE-MPs after an equilibrium that may be reached after 48

h in the 4th instar, resulting in a similar number of MPs inside the organisms on both studies and/or; iii) MPs have long residence times in the gut with slow or absent ingestion after some time that is dependent on the number of particles ingested reaching the maximal volumetric capacity of the digestive tract (SC I for concentration > 5g kg^{-1}) and; iv) a complete obstruction seems to be occurring after ingestion of (although fewer) particles of a higher diameter of size-classes II and III, since the number of particles ingested was not sufficient to reach the maximal volumetric capacity of the digestive tract of larvae. The long residence time of particles can be caused by either aggregation of particles inside the larval gut (most likely for the smaller-size particles – SC I) or due to blocking of the gut passage (probable when ingesting particles closer to their physiological size-limit of ingestion and/or reduced egestion – SC II and III). MPs' aggregation inside invertebrates' gut have already been observed and associated with higher gut retention time (Ogonowski et al., 2016). Also, recent research has shown a particularly extended residence time of similar size polystyrene microplastics (10-90 µm) within chironomids' gut needing more than 24 h to egest at least 50% of the ingested MPs (Scherer et al., 2017).

The fact that particles of SC II and III are not only larger (on average), but also more irregularly shaped, supports the idea of gut passage obstruction when compared to small and more spherical particles of SC I. Another indication of gut passage obstruction is the fact that the maximum number of particles ingested was reached for the lowest concentration tested of SC II (~785 particles) and III (~75 particles). Interestingly, AChE activity was significantly increased on larvae exposed to 20g kg^{-1} of PE-MPs of SC III, which is in good agreement with obstruction and inflammatory processes, since acetylcholine levels are known to

be increased in inflamed cells and tissues (De Oliveira et al., 2012; Gambardela et al., 2017). Moreover, an increase of peristaltic movements as an attempt to egest larger particles also explains the increased AChE activity observed in our current study. For SC I, the presence of MPs inside larval gut indicates a higher capacity for ingestion of smaller particles reaching a maximum number of ingested MPs of ~ 2400 for the highest concentration tested. Nevertheless, data also suggests that this number might be close to the maximal volumetric capacity of *C riparius* larval gut leading to increased retention time and potential aggregation, but not its severe obstruction.

Besides evidence of PE-MPs ingestion, retention and obstruction, our results show a significant increase of lipid peroxidation levels (LPO) on larvae of *C. riparius* exposed to all concentrations of all SC of PE-MPs, indicating that oxidative damage was triggered either by the high retention time of PE-MPs ingested (SC I) or obstruction (with potential inflammation) of their gut by big and irregular particles ingested when exposed to PE-MPs of SC II and III. Studies addressing biological effects of PE-MPs have pointed out that physical stress (rather than the chemical toxic effect of PE monomers) is the main toxicity-related factor (Von Moos et al., 2012; Wright et al., 2013b; Karami et al., 2016; Rehse et al., 2016) since an additional effort is needed to digest inert material and maintain physiological homeostasis (Von Moos et al., 2012; Karami et al., 2016). In fact, the persistence of MPs in the gut, like other particles (e.g., nanoparticles), was already described to induce a false sense of satiation and likely-causing inflammation, with consequent alterations on organisms feeding behaviour and physiological status (Von Moos et al., 2012; Wright et al., 2013a; Gall and Thompson, 2015). Inflammation-induced by PE-MPs retention in *C. riparius* larval

gut is the most probable cause for redox unbalance explaining the formation of reactive oxygen species (ROS) leading to oxidative damage, but further research concerning the immune response of larvae to MPs would be of great interest. Production of excessive ROS after exposure to MPs was previously reported in a variety of organisms, such as algae (Bhattacharya et al., 2010; González-Pleiter et al., 2019); copepods (Jeong et al., 2017); mussels (Von Moos et al., 2012; Paul-Pont et al., 2016), bivalves (Ribeiro et al., 2017), fish (Barboza et al., 2018), crustacea (Yu et al., 2018; González-Pleiter et al., 2019) and nematoda (Lei et al., 2018). The cascade prompted by the production of ROS, triggering oxidative stress and its related pathway is the main suggested MPs' toxicity mechanisms (Bhattacharya et al., 2010; Avio et al., 2015; Jeong et al., 2016, 2017; Paul-Pont et al., 2016; Alomar et al., 2017; Imhof et al., 2017; Veneman et al., 2017; Yu et al., 2018; Choi et al., 2018; Espinosa et al., 2018). Overall, ROS formation has also even been pointed out to be the key molecular initiating event of MPs toxicity (Jeong and Choi, 2019). Regardless of MPs size, effects triggered by the ingestion of particles by larvae *C. riparius* were severe. As explained, our results suggest that not only the number (SC I) but also the size of ingested particles (SC II and III) can mediate the oxidative stress effects observed in *C. riparius* larvae.

In addition to oxidative damage, evidence of deregulation in antioxidant defences was also found. First, a reduction of catalase (CAT) activity was observed in larvae exposed to PE-MPs (particularly evident for larvae that ingested larger particles). Catalase is involved in the removal of hydrogen peroxide (H_2O_2) (main precursor of hydroxyl radical), (Regoli and Giuliani, 2014) and similar inhibition of this enzyme was already observed for other invertebrates exposed to MPs (Paul-Pont et al., 2016). Authors suggested that CAT may follow

a biphasic response in the neutralisation of the H_2O_2 production after MPs ingestion (Paul-Pont et al., 2016). Likewise, it is possible that CAT activation occurs in the first hours/first day of exposure followed by a decrease in activity (Regoli et al., 2011). Nonetheless, other authors suggest that catalase is not actively involved in response to MPs in invertebrates (Avio et al., 2015; Ribeiro et al., 2017).

Second, glutathione-*S*-transferase (GST) activity was also decreased on larvae exposed to larger particles (SC III) which might indicate its inactivation. This pattern of inhibition of CAT and GST was previously reported for other invertebrate species exposed to microplastics (Avio et al., 2015; Ribeiro et al., 2017), suggesting that increased levels of ROS induced oxidative damage to proteins leading to enzyme inactivation.

Ingestion of non-nutritive particles and possible inflammation processes (with implications on oxidative stress and damage) observed here, can also imply energetic costs (Wright et al., 2013a). In the present study, the ingestion of PE-MPs induced energetic constraints to *C. riparius* larvae, namely by reducing lipid content (SC II and III) and the electron transport system (ETS) activity (all SC). The decrease of lipid reserves observed in the present study can be due not only to the inefficient and unprofitable digestion of non-nutritive particles (MPs) but also to energetic costs arising from physical stress and inflammation caused by large-size MPs. Like in the present study, other organisms such as marine worms presented depleted lipid reserves after MPs ingestion (Wright et al., 2013a). Besides, it has been shown in zebrafish larvae that MPs can activate a pathway for nuclear receptors involved in immunological recognition processes and lipid metabolism (Veneman et al., 2017). The decreased levels of lipids (and glycogen)

during the larval stage may have consequences on Chironomidae life-history traits (Hamburger et al., 1996; Silva et al., 2019). Moreover, the decreased ETS activity demonstrates impairment of aerobic energy production and is often associated with a decline in aerobic metabolism, which can lead to partial anaerobiosis and consequent effects at higher levels of biological organisation (Sokolova et al., 2012). A decrease on aerobic metabolism was also observed on fish (e.g., European seabass) after the ingestion of MPs, alongside an increased anaerobic metabolism (Barboza et al., 2018). Some organisms may use anaerobic pathways of energy production to get additional energy to face chemical stress or stress caused by the ingestion of MPs (Fırat et al., 2011; Oliveira et al., 2013). Moreover, we cannot exclude the possibility that partial damage to the inner mitochondrial membrane due to overproduction of ROS (Roche and Bogé, 1993; Stolze and Nohl, 1994; Choi et al., 2001) may have also impaired the electron transport system and be linked to the reduction of ETS activity. Either way, overall energy produced by larvae exposed to PE-MPs was not sufficient to cope with stress, since oxidative stress was unequivocally observed for all the SC tested.

The lack of mechanistic studies onto MPs toxicity has been reported as one of the priority knowledge gaps that urge to be addressed (Jeong and Choi, 2019). The present study provides an insight on the sub-cellular effects triggered by a high number of MPs ingested and retained within the gut of larvae of *C. riparius* after short-term exposure. In contrast to our findings, other studies report limited or no effects of microplastics on apical and sub-cellular endpoints (Imhof and Laforsch, 2016; Beiras et al., 2018; Weber et al., 2018; Castro et al., 2020; Khosrovyan and Kahru, 2020), but factors like organisms behaviour, polymer-

type, size of microplastics and mostly concentrations and exposure period have to be carefully taken into account for comparison of effects of microplastics. Moreover, the size of the mouth and digestive apparatus of each species used should also be carefully considered, since our results showed that this is an important factor for ingestion of particles (number and size of particles), retention time, obstruction and egestion. The concentrations of MPs used in this study are environmentally relevant (ranging from river hotspots to worst-case scenario) and induced biochemical effects that are associated with deleterious effects in the lifecycle (growth and development impairments) induced by PE-MPs in *C. riparius* (Silva et al., 2019). Thus, oxidative stress biomarkers and metabolic responses can be used as early warning indicators of acute stress-induced by microplastic' pollution, to compare the sensitivity of different species and assess harmful effects of different polymers that a are now emergent contaminants of aquatic ecosystems.

Roles

CS: conceptualization, investigation, data acquisition and analysis, writing - original draft, writing - review & editing. **ALPS**: funding aquisition, project administration, data aquisition and analysis, writing - review & editing. **DC**: investigation, data acquisition and analysis, writing - review & editing. **ALM**: data analysis, writing - review & editing; **JP:** conceptualization, supervision, writing - review & editing. **CG:** conceptualization, supervision, writing - review & editing.

Declaration of interests

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.

Acknowledgements

Thanks are due to FCT/MCTES for the financial support to CESAM (UIDP/50017/2020+UIDB/50017/2020), through national funds. This work was supported by the research project ComPET (POCI-01-0145-FEDER-030361), funded by FEDER, through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI), and by national funds (OE), through FCT/MCTES. CS and ALPS were supported by Portuguese Science Foundation (FCT) through scholarship SFRH/BD/128134/2016 and PD/BPD/114870/2016 + CEECIND/01366/2018, respectively; JLTP was supported by an FCT research contract (IF/01420/2015), and CG was supported by an FCT research contract (IF/01401/2014), all under POCH funds, co-financed by the European Social Fund and Portuguese National Funds from MEC. DC has a research contract within the project ComPET (nr. 5662).

References

Alomar, C., Sureda, A., Capó, X., Guijarro, B., Tejada, S., Deudero, S., 2017. Microplastic ingestion by *Mullus surmuletus* Linnaeus, 1758 fish and its potential for causing oxidative stress. Environ. Res. 159, 135–142. https://doi.org/10.1016/j.envres.2017.07.043

Armitage, P.D., Cranston, P.S., Pinder, L.C. V., 1995. The Chironomidae: biology and ecology of non-biting midges. Chapman and Hall.

ASTM, 1980. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. Am. Stand. Test. Mater. Annu. B. ASTM Stand. 46 E729-80 279–280.

Au, S.Y., Bruce, T.F., Bridges, WC, Klaine, SJ, 2015. Responses of *Hyalella azteca* to acute and chronic microplastic exposures. Environ. Toxicol. Chem. 34, 2564–2572. https://doi.org/10.1002/etc.3093

Avio, C.G., 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

Barboza, L.G.A., Vieira, L.R., Branco, V., Figueiredo, N., Carvalho, F., Carvalho, C., Guilhermino, L., 2018. Microplastics cause neurotoxicity, oxidative damage and energy-related changes and interact with the bioaccumulation of mercury in the European seabass, *Dicentrarchus labrax* (Linnaeus, 1758). Aquat. Toxicol. 195, 49–57. https://doi.org/10.1016/j.aquatox.2017.12.008

Beiras, R., Bellas, J., Cachot, J., Cormier, B., Cousin, X., Engwall, M., Gambardella, C., Garaventa, F., Keiter, S., Le Bihanic, F., López-Ibáñez, S., Piazza, V., Rial, D., Tato, T., Vidal-Liñán, L., 2018. Ingestion and contact with polyethylene microplastics does

not cause acute toxicity on marine zooplankton. J. Haz. Mat. 360, 452-460. https://doi.org/10.1016/j.jhazmat.2018.07.101

Bhattacharya, P., Lin, S., Turner, J.P., Ke, P.C., 2010. Physical adsorption of charged plastic nanoparticles affects algal photosynthesis. J. Phys. Chem. C 114, 16556–16561. https://doi.org/10.1021/jp1054759

Bird, R.P., Draper, H.H., 1984. Comparative Studies on Different Methods of Malonaldehyde Determination. Methods Enzymol. 105, 299–305. https://doi.org/https://doi.org/10.1016/S0076-6879(84)05038-2

Bradford, M.M., 1976. A Rapid and Sensitive Method for the Quantitation Microgram Quantities of Protein Utilising the Principle of Protein-Dye Binding 254, 248–254.

Castañeda, R.A., Avlijas, S., Simard, M.A., Ricciardi, A., 2014. Microplastic pollution in St Lawrence River sediments. Can. J. Fish. Aquat. Sci. 71, 1767–1771. https://doi.org/10.1007/978-3-319-61615-5_12

Castro, G., Bernegossi, A. C., Felipe, M.C., Corbi, J. J., 2020. Is the development of Daphnia magna neonates affected by short-term exposure to polyethylene microplastics? Journal of Environmental Science and Health, Part A, 55:8, 935-946. https://doi.org/10.1080/10934529.2020.1756656

Chen, Q., Gundlach, M., Yang, S., Jiang, J., Velki, M., Yin, D., Hollert, H., 2017. Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity. Sci. Total Environ. 584–585, 1022–1031. https://doi.org/10.1016/j.scitotenv.2017.01.156

Choi, J., Roche, H., Caquet, T., 2001. Hypoxia, hyperoxia and exposure to potassium dichromate or fenitrothion alter the energy metabolism in *Chironomus riparius* Mg. (Diptera: Chironomidae) larvae. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 130, 11–17. https://doi.org/10.1016/S1532-0456(01)00206-X

Choi, J.S., Jung, Y.J., Hong, N.H., Hong, S.H., Park, J.W., 2018. Toxicological effects of irregularly shaped and spherical microplastics in a marine teleost, the sheepshead minnow (*Cyprinodon variegatus*). Mar. Pollut. Bull. 129, 231–240. https://doi.org/10.1016/j.marpolbul.2018.02.039

Claiborne, A., 1985. Catalase activity, in: Greenwald, R.A. (Ed.), CRC Handbook of Methods for Oxygen Radical Research. Boca Raton, pp. 283–284.

Coen, W.M. De, Janssen, C.R., 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed *Daphnia* populations. J. Aquat. Ecosyst. Stress Recover. 6, 43–55. https://doi.org/https://doi.org/10.1023/A:1008228517955

Conkle, J.L., Báez Del Valle, C.D., Turner, J.W., 2017. Are We Underestimating Microplastic Contamination in Aquatic Environments? Environ. Manage. 61. https://doi.org/10.1007/s00267-017-0947-8

Corcoran, P.L., 2015. Benthic plastic debris in marine and fresh water environments. Environ. Sci. Process. Impacts 17, 1363–1369. https://doi.org/10.1039/c5em00188a

Deng, Y., Zhang, Y., Lemos, B., Ren, H., 2017. Tissue accumulation of microplastics in mice and biomarker responses suggest widespread health risks of exposure. Sci. Rep. 7, 46687. https://doi.org/10.1038/srep46687

Ellman, G.L., Courtney, K.D., Andres, V.J., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95. https://doi.org/https://doi.org/10.1016/0006-2952(61)90145-9

Epler, J.H., 1995. Identification manual for the larvae Chironomidae (Diptera) of Florida. Department of Environmental Protection, Tallahassee, FL.

Espinosa, C., García Beltrán, J.M., Esteban, M.A., Cuesta, A., 2018. In vitro effects of

virgin microplastics on fish head-kidney leucocyte activities. Environ. Pollut. 235, 30–38. https://doi.org/10.1016/j.envpol.2017.12.054

Fırat, Ö., Cogun, H.Y., Yüzereroğlu, T.A., Gök, G., Firat, Ö., Kargin, F., Kötemen, Y., 2011. A comparative study on the effects of a pesticide (cypermethrin) and two metals (copper, lead) to serum biochemistry of Nile tilapia, *Oreochromis niloticus*. Fish Physiol. Biochem. 37, 657–666. https://doi.org/10.1007/s10695-011-9466-3

Frias, JPGL, Nash, R., 2019. Microplastics: Finding a consensus on the definition. Mar. Pollut. Bull. 138, 145–147. https://doi.org/10.1016/j.marpolbul.2018.11.022

Gall, S.C., Thompson, R.C., 2015. The impact of debris on marine life. Mar. Pollut. Bull. 92, 170–179. https://doi.org/10.1016/j.marpolbul.2014.12.041

Gardon, T., Reisser, C., Soyez, C., Quillien, V., Le Moullac, G., 2018. Microplastics Affect Energy Balance and Gametogenesis in the Pearl Oyster *Pinctada margaritifera*. Environ. Sci. Technol. 52, 5277–5286. https://doi.org/10.1021/acs.est.8b00168

Grelli, K.N., M. Palubinsky, A., Cozette Kale, A., Lizama-Manibusan, B.N., N. Stankowski, J., L. Milne, G., Singer, R., McLaughlin, B., 2013. Alteration of Isocitrate Dehydrogenase Following Acute Ischemic Injury as a Means to Improve Cellular Energetic Status in Neuroadaptation. CNS Neurol. Disord. - Drug Targets 12, 849–860. https://doi.org/10.2174/18715273113129990062

González-Pleiter, M.,Tamayo-Belda, M., Pulido-Reyes G., Amariei G., Leganés, F., Rosal, R., Fernández-Piñas, F., 2019. Secondary nanoplastics released from a biodegradable microplastic severely impact freshwater environments. Environ. Sci. Nano 6, 1382-1392. https://doi.org/ 10.1039/C8EN01427B.

Guilhermino, L., Lopes, M.C., Carvalho, A.P., Soares, AMVM, 1996. Acetylcholinesterase Activity in Juveniles of *Daphnia magna* Straus. Bull. Environ. Contam. Toxicol. 57, 979–985. https://doi.org/10.1007/s001289900286

Habig, W.H.H., Pabst, M.J.J., Jacoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7140.

Hamburger, K., Lindegaard, C., Dall, P.C., 1996. The role of glycogen during the ontogenesis of *Chironomus anthracinus* (Chironomidae, Diptera). Hydrobiologia 318, 51–59. https://doi.org/10.1007/bf00014131

Henriques-Oliveira, a L., Nessimian, J.L., Dorvillé, L.F., 2003. Feeding habits of chironomid larvae (Insecta: Diptera) from a stream in the Floresta da Tijuca, Rio de Janeiro, Brazil. Braz. J. Biol. 63, 269–281. https://doi.org/10.1590/S1519-69842003000200012

Hurley, R., Woodward, J., Rothwell, J.J., 2018. Microplastic contamination of river beds significantly reduced by catchment-wide flooding. Nat. Geosci. 11, 251–257. https://doi.org/10.1038/s41561-018-0080-1

Imhof, H.K., Laforsch, C., 2016. Hazardous or not - Are adult and juvenile individuals of Potamopyrgus antipodarum affected by non-buoyant microplastic particles? Environ. Pollut. 218, 383–391. https://doi.org/10.1016/j.envpol.2016.07.017

Imhof, H.K., Rusek, J., Thiel, M., Wolinska, J., Laforsch, C., 2017. Do microplastic particles affect *Daphnia magna* at the morphological, life history and molecular level? PLoS One 12, 1–20. https://doi.org/10.1371/journal.pone.0187590

Jeong, C.-B., Kang, H.-M., Lee, M.-C., Kim, D.-H., Han, J., Hwang, D.-S., Souissi, S., Lee, S.-J., Shin, K.-H., Park, H.G., Lee, J.-S., 2017. Adverse effects of microplastics and oxidative stress-induced MAPK/Nrf2 pathway-mediated defense mechanisms in the marine copepod *Paracyclopina nana*. Sci. Rep. 7, 41323. https://doi.org/10.1038/srep41323

Jeong, C.B., Won, E.J., Kang, H.M., Lee, M.C., Hwang, D.S., Hwang, U.K., Zhou, B., Souissi, S., Lee, S.J., Lee, J.S., 2016. Microplastic Size-Dependent Toxicity,

Oxidative Stress Induction, and p-JNK and p-p38 Activation in the Monogonont Rotifer (*Brachionus koreanus*). Environ. Sci. Technol. 50, 8849–8857. https://doi.org/10.1021/acs.est.6b01441

Jeong, J., Choi, J., 2019. Adverse outcome pathways potentially related to hazard identification of microplastics based on toxicity mechanisms. Chemosphere 231, 249–255. https://doi.org/10.1016/j.chemosphere.2019.05.003

Jin, Y., Xia, J., Pan, Z., Yang, J., Wang, W., Fu, Z., 2018. Polystyrene microplastics induce microbiota dysbiosis and inflammation in the gut of adult zebrafish. Environ. Pollut. 235, 322–329. https://doi.org/10.1016/j.envpol.2017.12.088

Karami, A., Romano, N., Galloway, T., Hamzah, H., 2016. Virgin microplastics cause toxicity and modulate the impacts of phenanthrene on biomarker responses in African catfish (*Clarias gariepinus*). Environ. Res. 151, 58–70. https://doi.org/10.1016/j.envres.2016.07.024

Khosrovyan, A., Gabrielyan, B., Kahru, A., 2020. Ingestion and effects of virgin polyamide microplastics on *Chironomus riparius* adult larvae and adult zebrafish *Danio rerio*. Chemosphere 259, 127456. https://doi.org/10.1016/j.chemosphere.2020.127456

Khosrovyan, A., Kahru, A., 2020. Evaluation of the hazard of irregularly-shaped co-polyamide microplastics on the freshwater non-biting midge *Chironomus riparius* through its life cycle. Chemosphere 244, 125487. https://doi.org/10.1016/j.chemosphere.2019.125487

Klein, S., Worch, E., Knepper, T.P., 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

Lagarde, F., Zanella, M., Daniel, P., Hiard, S., 2016. Microplastic interactions with

freshwater microalgae: Hetero-aggregation and changes in plastic density appear strongly dependent on polymer type. Environ. Pollut. 215, 331–339. https://doi.org/10.1016/j.envpol.2016.05.006

Lambert, S., Scherer, C., Wagner, M., 2017. Ecotoxicity Testing of Microplastics: Considering the Heterogeneity of Physicochemical Properties. Integr. Environ. Assess. Manag. 13, 470–475. https://doi.org/10.1002/ieam.1901

Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., Shi, H., Raley-Susman, K.M., He, D., 2018. Microplastic particles cause intestinal damage and other adverse effects in zebrafish *Danio rerio* and nematode *Caenorhabditis elegans*. Sci. Total Environ. 619–620, 1–8. https://doi.org/10.1016/j.scitotenv.2017.11.103

Li, C., Busquets, R., Campos, L.C., 2019. Assessment of microplastics in freshwater systems: A review. Sci. Total Environ. 707, 135578. https://doi.org/10.1016/j.scitotenv.2019.135578

Lu, L., Wan, Z., Luo, T., Fu, Z., Jin, Y., 2018. Polystyrene microplastics induce gut microbiota dysbiosis and hepatic lipid metabolism disorder in mice. Sci. Total Environ. 631–632, 449–458. https://doi.org/10.1016/j.scitotenv.2018.03.051

Lu, Y., Zhang, Y., Deng, Y., Jiang, W., Zhao, Y., Geng, J., Ding, L., Ren, H., 2016. Uptake and Accumulation of Polystyrene Microplastics in Zebrafish (*Danio rerio*) and Toxic Effects in Liver. Environ. Sci. Technol. 50, 4054−4060. https://doi.org/10.1021/acs.est.6b00183

Luís, L.G., Ferreira, P., Fonte, E., Oliveira, M., Guilhermino, L., 2015. Does the presence of microplastics influence the acute toxicity of chromium (VI) to early juveniles of the common goby (*Pomatoschistus microps*)? A study with juveniles from two wild estuarine populations. Aquat. Toxicol. 164, 163–174. https://doi.org/10.1016/j.aquatox.2015.04.018

Luís, L.G., Guilhermino, L., 2012. Short-term toxic effects of naphthalene and pyrene on the common prawn (*Palaemon serratus*) assessed by a multi-parameter laboratorial approach: Mechanisms of toxicity and impairment of individual fitness. Biomarkers 17, 275–285. https://doi.org/10.3109/1354750X.2012.666765

Lusher, A.L., Welden, N.A., Sobral, P., Cole, M., 2017. Sampling, isolating and identifying microplastics ingested by fish and invertebrates. Anal. Methods 9, 1346–1360. https://doi.org/10.1039/c6ay02415g

Nel, H.A., Dalu, T., Wasserman, R.J., 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

Ogonowski, M., Schür, C., Jarsén, Å., Gorokhova, E., 2016. The Effects of Natural and Anthropogenic Microparticles on Individual Fitness in *Daphnia magna*. PLoS One 11, 1–20. https://doi.org/10.1371/journal.pone.0155063

Oliveira, M., Ribeiro, A., Hylland, K., Guilhermino, L., 2013. Single and combined effects of microplastics and pyrene on juveniles (0+ group) of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). Ecol. Indic. 34, 641–647. https://doi.org/10.1016/j.ecolind.2013.06.019

Paul-Pont, I., Lacroix, C., González Fernández, C., Hégaret, H., Lambert, C., Le Goïc, N., Frère, L., Cassone, A.L., Sussarellu, R., Fabioux, C., Guyomarch, J., Albentosa, M., Huvet, A., Soudant, P., 2016. Exposure of marine mussels *Mytilus* spp. to polystyrene microplastics: Toxicity and influence on fluoranthene bioaccumulation. Environ. Pollut. 216, 724–737. https://doi.org/10.1016/j.envpol.2016.06.039

Redondo-Hasselerharm, P.E., Gort, G., Peeters, ETHM, Koelmans, A.A., 2020. Nano- and microplastics affect the composition of freshwater benthic communities in the

long term. Sci. Adv. 6. https://doi.org/10.1126/sciadv.aay4054

Regoli, F., Giuliani, M.E., 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

Regoli, F., Giuliani, M.E., Benedetti, M., Arukwe, A., 2011. Molecular and biochemical biomarkers in environmental monitoring: A comparison of biotransformation and antioxidant defense systems in multiple tissues. Aquat. Toxicol. 105, 56–66. https://doi.org/10.1016/j.aquatox.2011.06.014

Rehse, S., Kloas, W., Zarfl, C., 2016. Short-term exposure with high concentrations of pristine microplastic particles leads to immobilisation of *Daphnia magna*. Chemosphere 153, 91–99. https://doi.org/10.1016/j.chemosphere.2016.02.133

Ribeiro, F., Garcia, A.R., Pereira, B.P., Fonseca, M., Mestre, N.C., Fonseca, T.G., Ilharco, L.M., Bebianno, M.J., 2017. Microplastics effects in *Scrobicularia plana*. Mar. Pollut. Bull. 122, 379–391. https://doi.org/10.1016/j.marpolbul.2017.06.078

Ristola, T., Kukkonen, J.V.K., Pellinen, J., 1999. Body Residues and Responses of the Midge *Chironomus riparius* to Sediment-Associated 2,4,5-Trichlorophenol in Subchronic and Chronic Exposures. Arch. Environ. Contam. Toxicol. 37, 42–49. https://doi.org/https://doi.org/10.1007/s002449900488

Roche, H., Bogé, G., 1993. Effects of Cu, Zn and Cr salts on antioxidant enzyme activities in vitro of red blood cells. Toxic. Vitr. 7, 623–629. https://doi.org/https://doi.org/10.1002/etc.5620130320

Rodrigues, A.C.M., Gravato, C., Quintaneiro, C., Golovko, O., Vladimír, Ž., Barata, C., Soares, AMVM, Pestana, J.L.T., 2015. Life history and biochemical effects of chlorantraniliprole on *Chironomus riparius*. Sci. Total Environ. 508, 506–513. https://doi.org/10.1016/j.scitotenv.2014.12.021

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

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. 134604. https://doi.org/10.1016/j.scitotenv.2019.134604

Silva, C.J.M., Patrício, A.L., Gravato, C., Pestana, J.L.T., 2019. Science of the Total Environment 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

Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar. Environ. Res. 79, 1–15. https://doi.org/10.1016/j.marenvres.2012.04.003

Stanković, J., Milošević, D., Savić-Zdraković, D., Yalçın, G., Yildiz, D., Beklioğlu, M., Jovanović, B., 2020. Exposure to a microplastic mixture is altering the life traits and is causing deformities in the non-biting midge *Chironomus riparius* Meigen (1804). Environ. Pollut. 262, 114248. https://doi.org/10.1016/j.envpol.2020.114248

Stolze, K., Nohl, H., 1994. Effect of xenobiotics on the respiratory activity of rat heart mitochondria and the concomitant formation of superoxide radicals. Environ. Toxicol. Chem. 13, 499–502. https://doi.org/https://doi.org/10.1002/etc.5620130320

Syrovátka, V., Schenková, J., Brabec, K., 2009. The distribution of chironomid larvae and oligochaetes within a stony-bottomed river stretch: the role of substrate and

hydraulic characteristics. Fundam. Appl. Limnol. 174, 43–62. https://doi.org/10.1127/1863-9135/2009/0174-0043

Veneman, W.J., Spaink, H.P., Brun, N.R., Bosker, T., Vijver, M.G., 2017. Pathway analysis of systemic transcriptome responses to injected polystyrene particles in zebrafish larvae. Aquat. Toxicol. 190, 112–120. https://doi.org/10.1016/j.aquatox.2017.06.014

Von Moos, N., Burkhardt-Holm, P., Köhler, A., 2012. Uptake and effects of microplastics on cells and tissue of the blue mussel *Mytilus edulis* L. after an experimental exposure. Environ. Sci. Technol. 46, 11327–11335. https://doi.org/10.1021/es302332w

Weber, A., Scherer C., Brennholt N., Reifferscheid G., Wagner M., 2018. PET microplastics do not negatively affect the survival, development, metabolism and feeding activity of the freshwater invertebrate *Gammarus pulex.* Environ. Pollut. 234, 181-189. https://doi.org/10.1016/j.envpol.2017.11.014

Wright, S.L., Rowe, D., Thompson, R.C., Galloway, T.S., 2013a. Microplastic ingestion decreases energy reserves in marine worms. Curr. Biol. 23, 1031–1033. https://doi.org/10.1016/j.cub.2013.10.068

Wright, S.L., Thompson, R.C., Galloway, T.S., 2013b. The physical impacts of microplastics on marine organisms: a review. Environ. Pollut. 178, 483–492. https://doi.org/10.1016/j.envpol.2013.02.031

Yu, P., Liu, Z., Wu, D., Chen, M., Lv, W., Zhao, Y., 2018. Accumulation of polystyrene microplastics in juvenile *Eriocheir sinensis* and oxidative stress effects in the liver. Aquat. Toxicol. 200, 28–36. https://doi.org/10.1016/j.aquatox.2018.04.015

Ziajahromi, S., Kumar, A., Neale, P.A., Leusch, F.D.L., 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