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Ingestion of small-sized and irregularly shaped polyethylene microplastics affect *Chironomus riparius* life-history traits

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Abstract

Microplastics (MPs) are emerging contaminants of freshwater ecosystems. Once in aquatic systems, most of these plastic particles undergo processes of fragmentation, biofouling, and sedimentation, resulting in increased concentrations of smaller sized and irregularly-shaped particles in the sediment. High levels of MPs in freshwater sediments can denote a potential threat to benthic and sediment-dwelling organisms such as dipteran larvae. This study evaluates the ecotoxicological effect of three pools of irregularly-shaped polyethylene microplastics (pools containing 90% of the particles within 32-63 µm (size-class A), 63-250 µm (size-class B) and 125-500 µm (size-class C)), with concentrations ranging from 1.25 to 20 g Kg^{-1} sediment, on the dipteran *Chironomus riparius* life-history traits. After ten days of exposure, larvae ingested PE particles typically in the 32-63 µm range, even when 90% of the particles possessed higher size (i.e., in size-classes B and C) and the larvae mandible allowed the ingestion of such bigger-sized particles. Thus, the number of ingested particles was higher in size-class A, followed by B and C, and led to a significant reduction with similar magnitude on larval growth (Lowest Observed Effect Concentrations (LOEC)=2.5 g Kg^{-}

^{1} sediment DW) and a significant delay on imagoes emergence (e.g. LOEC = 1.5 g Kg^{-1} sediment DW for females).

The results from this study show that the ingestion and persistence of small-sized polyethylene microplastics caused significant impairments on life-history traits of *C. riparius.* Considering their role on freshwater food-webs and the potential persistence of small-sized PE particles in their larval gut, these results also point for the potential adverse effects of small-sized microplastics at the community and ecosystem level.

Keywords: benthic macroinvertebrates, freshwaters, insects growth, emergence; pollution

1. Introduction

Microplastics (MPs, < 5mm in size) are an emergent environmental issue, and its research is rapidly advancing (Jiang, 2017; Alimi et al., 2018; Chae and An, 2018). Recent attention was driven towards primary MPs, i.e., plastic particles manufactured at microscale (e.g., microbeads, capsules, fibers, pellets) that are being released continuously into the freshwater environments via sewage, runoff from inland activities, illegal industrial discharge and wind (Boucher and Friot, 2017). Once in the water systems, MPs are likely to undergo through processes of disintegration, fragmentation, and biofouling, resulting into a wide range of smaller particles that either remain in the water column or mainly settle in the freshwater sediments (Castañeda et al., 2014; Lagarde et al., 2016). Documented as important pathways and repository of plastic debris (GESAMP, 2015, 2016), freshwater compartments can contain MPs in concentrations in the order of grams of MP particles per Kg/ cubic meter, or thousands of MP particles per kg/ cubic meter of sediment (Klein et al., 2015; Hurley et al., 2018; Rodrigues et al., 2018; Wang et al., 2019) which can be directly comparable to concentrations of MP's found in shoreline ecosystems (McCormick et al., 2014; Dris et al., 2015; Anderson et al., 2017).

Despite freshwater sediments being recognized as central repositories of MPs (Hurley et al., 2018), most of the ecotoxicological studies performed so far have been focused on filtering invertebrates, such as daphniids and mussels (Ogonowski et al., 2016; Guilhermino et al., 2018), and fish (Silva-Cavalcanti et al., 2017), which are potentially exposed to buoyant MPs (Eerkes-Medrano et al., 2015; Ziajahromi et al., 2017). The few available studies on freshwater benthic invertebrates (mostly using oligochaete, amphipods and dipteran larvae) reported the ingestion of small MPs (size < 300 µm). Along with the ingestion, these studies also point some evidences of higher retention time of microplastics in their gut when compared to other freshwater organisms and even to marine sediment-dwelling invertebrates (Courtene-Jones et al.,

2017; Scherer et al., 2017; Al-jaibachi and Callaghan, 2018; Lei et al., 2018; Ziajahromi et al., 2018). In laboratory assays, the presence of MP particles has been shown to induce harmful effects on feeding and development in dipteran larvae (Scherer et al., 2017; Al-jaibachi et al., 2018; Ziajahromi et al., 2018). However, these investigations focused only on MPs with “bead” shape of specific sizes. In natural environments, MP beads are likely to undergo processes of degradation, eventually fragment and disintegrate forming particles consisting of a broad particle size distribution with a diverse range of particle shapes (Lambert et al., 2017). Furthermore, non-spherical MP shapes may cause higher toxicity than microbeads, as reported for marine amphipods (Au et al., 2015).

The non-biting midge larvae *Chironomus riparius* is one of the most abundant macroinvertebrate species in freshwater benthic ecosystems (Armitage et al., 1995). Inhabiting the uppermost layers of sediment, they act as deposit-feeders feeding on sedimented and deposited organic matter. Their feeding behavior is mostly non-selective determined by bioavailability, and (more rarely) selective, based typically on the nutritive value and food type (Rasmussen, 1984; Armitage et al., 1995). In sediments contaminated with MPs, we hypothesize that larvae will likely ingest such synthetic polymers as they ingest natural sediments and particulate organic matter (detritus) of similar size, resulting in growth and emergence impairments. Nevertheless, such ingestion will likely be dependent on the larvae pre-mandible width and the size-range of the MPs present in the sediments. Typically, first instar larvae of *Chironomus riparius* ingest sediment particles up to 20 μm as part of their regular feeding activity. Conversely, final instars (3^{rd}-4^{th} instar) can ingest particles typically in the 60-200 μm fraction (Armitage et al., 1995; Ristola et al., 1999; Henriques-Oliveira et al., 2003). Therefore, the objectives of this study are: 1) to determine which size-range are preferably ingested by *C. riparius* larvae in the presence of a pool of particles of different size; 2) to evaluate if the ingestion is dose and pool dependent, and 3) to assess the ecotoxicological effects of MPs on *C. riparius* life history traits (growth and

emergence). Thus, *C. riparius* larvae were exposed to increased concentrations of three size-classes (pools) of irregularly shaped polyethylene particles (PE, one of the most produced primary MP, Conkle et al., 2017) and effects of their ingestion on growth and emergence followed throughout their life cycle (~28 d) according to OECD guidelines.

2. Materials and methods

2.1. Test species and culture conditions

Chironomus riparius used in the bioassays were obtained from a culture established at the Department of Biology, University of Aveiro. Organisms are maintained in controlled conditions (16:8 h light-dark photoperiod, temperature of 20 ± 1°C), with the larvae growing in 30×20 cm polypropylene containers, and adults constrained within a 120×60×40 cm acrylic cage. The polypropylene containers contain a layer of approximately 3 cm of previously burnt (500 °C for 4h) inorganic fine sediment (<1 mm). The containers also contain American Society for Testing Materials (ASTM) hard water in 1:4 ratio (ASTM, 1980). Feeding for larvae is constituted by a suspension of macerated TetraMin (Tetrawerke, Melle, Germany) that is provided every two days (*ad libitum*). The medium (ASTM hard water) is changed every two weeks and the sediment every month. The life cycle of *C. riparius* in laboratory-controlled conditions is completed within 3-4 weeks.

Before experiments, egg ropes were collected and transferred to 50 mL glass vessels filled with ASTM hard water. First instar larvae (less than 48 h post-hatching) were used for the tests.

2.2. Polyethylene particles used for testing

Commercially available polyethylene (PE) particles of three different size classes were purchased: 40-48 µm (average size, ultra-high molecular weight powder, CAS No. 9002-88-4, Sigma-Aldrich UK); 125 µm (average size, ultra-high molecular weight powder, CAS Number 9002-88-4, Sigma-Aldrich UK) and 350 µm (average size, medium-density polyethylene powder, CAS 708-316-83, Goodfellow). The size range of the polyethylene particles was chosen based on the size of typical food items ingested by the larvae of *C. riparius* (Epler, 1995; Ristola et al., 1999; Henriques-Oliveira et al., 2003). Particle size distribution of the purchased particles was

determined by vibratory sieve shaking (mesh pore-sizes: 500, 250, 125, 63 and 32 µm). Replicates of 100 g of PE particles of each size class were sieved in a vibratory sieve shaker for particle size analysis within each size-class. The purchased PE particles presented a pool of particles with different sizes (Table 1). To avoid misleading, the results are presented according to three size-class: A, B, and C; characterized by ≥ 90% of the particles with a size range of 32-63 µm, 63-250 µm and 125-500 µm, respectively.

All equipment and test vials were acid washed and thoroughly rinsed with Milli-Q water before use in the tests. Plastic contamination via air exposure was minimized by covering samples and filters. Glassware was used instead of plasticware whenever possible. For MPs extraction and quantification, additional controls were used to account for possible airborne MPs contamination. The average blanks/controls was then subtracted from the particle quantification to correct for background contamination.

2.3. Polyethylene concentrations and sediment spiking

The tested concentrations were: 1.25, 2.5, 5, 10, 20 g PE Kg^{-1} sediment, for all the different PE particles. The concentrations range was set based on recent estimations of primary MPs (size < 300 µm) on freshwaters hotspots (Conkle et al., 2017).

For each treatment, PE particles were directly mixed into the sediment (< 1mm, previously burnt at 500 °C). Each glass test vial contained 50 g of control (sediment with no MPs) or 50g of sediment mixed with MPs and filled with ASTM hard water (~150 mL). The addition of ASTM hard water was performed by gently pouring the ASTM hard water to minimize resuspension of PE particles. The test vials were then covered with lids and allowed to equilibrate for 24 h.

2.4. *Chironomus riparius* partial life cycle test

First instar (less than 48 h post-hatching) *C. riparius* larvae were used in chronic 28-days partial life cycle assays (OECD, 2004). Each treatment condition and a control (uncontaminated sediment) consisted of thirteen replicates with five larvae each. During the experiment, organisms were fed every two days (0.5 mg of macerated TetraMin per organism per day), and the test conditions were the same as described for culturing. After ten days, larvae from five replicates of each treatment were sacrificed in ethanol 70%, counted, and measured (total length) using a stereo dissecting microscope fitted with a calibrated eyepiece micrometer. Larvae from three replicates of each treatment were carefully rinsed (three times) in Milli-Q water, checked under a stereoscopic microscope (for MP adhered to the integument), and frozen at -20°C for MPs quantification in the organisms. The remaining five replicates (of the initial thirteen) were used to follow the emergence of emerged adult insects (imagoes) until the end of the test. Imagoes were daily collected from emergence traps and placed in 5 mL tubes with ethanol 70% for correct identification of the sex (male/female). Water quality parameters (pH, oxygen dissolved and conductivity) were evaluated every three days.

2.5. Extraction and quantification of PE particles in sediment, water and in chironomids

The quantification of PE particles in sediment and water column was performed for the two lowest concentrations (1.25 and 2.5 g Kg^{-1} sediment) before exposure to ensure real concentrations were not misrepresented comparing with nominal concentrations. Before the quantification of resuspended PE particles in the water column, the buoyant PE particles present in the water column were collected and filtered using pre-weighted (iW) black polycarbonate filters (PCTE, 0.2 μm pore size, 42 mm ∅, ref. 7063-4702, GE Healthcare Whatman™) to retain microplastics.

The extraction of PE particles present in the sediment followed the principle of density separation reported by Thompson et al., 2004, based on supersaturated NaCl solution (NaCl/ MilliQ, density ρ=1.2 g mL^{-1}) and a customized glassware designed by Karlsson and colleagues (Karlsson et al., 2017) onto pre-weighted PCTE filters.

After vacuum-filtration, all filters (from water and sediment samples) were treated with H_2O_2 (30%) to eliminate possible organic matter, dried at 25 ºC for 2-3 days, and weighed again (fW). The mass of PE particles in the sediment and resuspended in water was estimated by measuring the difference between the final (fW) and the initial weight (iW) of PCTE filters (using weight of blank filters for account possible microplastics' airborne contamination).

After exposure, the extraction and quantification of MPs from biological samples followed previous recommendations (Lusher et al., 2017). Briefly, biological samples were freeze-dried for 24h and weighed (dry weight, DW). Samples were transferred to glass flasks and carefully grounded with a small glass rod (which acted as a small mortar and pestle) to facilitate further digestion with 3 mL of HNO_3 for three hours at 60 °C. After cooling down to room temperature (RT), 2.6 mL of H_2O_2 (35%) was added to complete the digestion. After 24h, and if no visible oxygen bubbles were being released, samples were then gently diluted 1:10 with Milli-Q (at RT) and vacuum-filtered onto black PCTE filters. Retained biological material (in the filtration apparatus) was copiously flushed with Milli-Q. Membranes were transferred to glass petri-dish and allowed to dry in the oven at 25 °C for 2-3 days. Afterward, the number of particles ingested by the larvae were counted under a stereomicroscope. To assess the average size of the particles ingested by *C. riparius* larvae, the diameter (major axis when particles were fitted to an ellipse) of all particles found in each filter of the lowest concentration tested for all size-class was measured under a stereomicroscope (stereoscopic zoom microscope—SMZ 1500, Nikon Corporation) associated to NIS-Elements D 3.2 microscope imaging software.

2.6. Statistical analysis

Effects of PE-particles exposure on *C. riparius* growth were analyzed using parametric analysis of variance (ANOVA) with multiple comparisons examined by Dunnett's post hoc test. Normality of data and homogeneity of variances were assessed by performing Shapiro-Wilk and Levene's test, respectively. In case of non-normal distribution and the heterogeneous variances of the data set, non-parametric analysis was performed, i.e., Mann-Whitney tests and Kruskal-Wallis tests with Dunn's multiple comparison tests to assess significant differences.

Based on the time and the number of emerged imagoes, the mean emergence time (EmT_{50}) of *C. riparius* was calculated for each condition. The natural logarithm of time (in days) was taken, and the number of emerged imagoes was cumulated and normalized to percentages for each replicate. The mean emergence time (EmT_{50}) was determinate through dose-response analysis (survival curve), model Eq. Y=Bottom + (Top-Bottom)/(1– 10^((LogEC_{50}-x)*HillSlope)), and was statistically compared according to (Sprague and Fogels, 1976), based on Wilcoxon two-group test. The sex ratio of imagoes was calculated in each treatment as the number of males divided by the number of female organisms. A chi-square test was applied to evaluate the effect of dose and size of PE particles in sex ratio.

For all statistical tests, the significance level was set at $p<0.05$. Data were analyzed using GraphPad 7 (GraphPad Software Inc., La Jolla California USA), except for EmT_{50} values that were calculated with the statistical software R Ver 3.5.1. from The R Foundation for Statistical Computing (Vienna, Austria), using the "drc" package.

3. Results

3.1. Polyethylene characteristics and concentrations in sediment, water, and whole organisms

At the beginning of the exposure, more than 98% of polyethylene (PE) particles remained in the sediment while less than 2% resuspended in the water column (table 2).

After ten days of exposure, larvae presented PE particles in their gut in all tested PE size-class (Figure 1A, B and C). Ingestion was size dependent, with larvae exposed to size-class A revealing higher internal PE concentrations than larvae exposed to size-class B and C ($F_{2,20}$ = 15.13, $p<0.001$). Besides, for larvae exposed to PE size-class A, significant dose-related ingestion of PE particles was observed ($p<0.05$), with larvae exposed to higher PE concentrations presenting more PE particles in their gut. The average size of the ingested particles was lower than the average size of the particles contained in the sediment (40.93 ± 0.34 µm, 57.08 ± 0.80 µm and 60.82 ± 1.59 µm for PE size-class A, B, and C, respectively, Figure 1D). The average size of the ingested particles corresponded to the fraction 32-63 µm, which in the size-class A included more than 90% or the particles, and in size-class B and C integrated less than 10% of the particles present.

3.2. Life-history effects

During exposure the water quality parameters remained in the recommended range defined by OECD (OECD, 2004): temperature of 20.6 ± 0.6 ºC, pH at 8.34 ± 0.07, air saturation values between 70% and 90%. The survival and emergence of adults in control conditions were above 80%.

The effects of the ingestion of PE particles on larval growth can be observed in Figure 2. The ingestion of PE particles of all size-classes significantly reduced larval growth after 10 days of exposure in comparison with the control treatment (size class A: $F_{(5,23)}$ =61.417, p<0.001; size class B: $F_{(5,22)}$ =10.943, p<0.001; and size class C: $F_{(5,24)}$ =11.378, p<0.001). Stronger effects were observed for the smaller-sized PE class (size-class A) which caused a 10% reduction in larval length from the second tested concentration (2.5 g PE kg^{-1} DW sediment), and a 40% reduction at the highest tested concentration (20 g PE kg^{-1} DW sediment). For size-class B and C, significant effects were observed at the two highest concentrations tested with 9% and 11% reductions in larval growth at 10 g PE kg^{-1} DW sediment for size-class B and C, respectively; and 17% and 20% for 20 g PE kg^{-1} DW sediment for size-class B and C, respectively.

The mean time to emergence (EmT_{50}) of imagoes in control conditions was around 16 days (males: 15.3±0.1; females: 16.6±0.1) (Figure 3; Table S1). In the presence of PE particles, a significant delay was observed with stronger effects for the size-class A (32-63 µm). The significant delay on females' emergence induced by these smaller sized particles was observed right from the first concentration tested (1.25 g Kg^{-1} sediment DW) while in males it was only observed from the third tested concentration (5 g Kg^{-1} sediment DW). The typical *C. riparius* emergence pattern, with females emerging consistently later than males, was observed in control (≈1 day) and PE treatments (≈2-4 days). The difference in time to emergence between males and females increased (2.1-2.5 times higher) for the two lower PE concentrations (1.25 and 2.5 g PE kg^{-1} sediment) of size class A and B.

There was no effect of PE particles size and concentration on the male/female ratio (chi-square tests, Supplementary data).

4. Discussion

Microplastics (MPs) are emerging contaminants of freshwater sediments and may impose a risk to benthic invertebrate species. As initially hypothesized, *Chironomus riparius* ingested polyethylene particles present in sediments with size- and dose-related adverse effects on development (growth and emergence). Moreover, we observed an accumulation of small-sized particles (40-60 μm) in the gut of larvae. These results confirm the ingestion of polyethylene (and possibly of any king of MP) and stress the need to monitor their presence in freshwater sediments and potential ecological effects.

The predominant non-selective feeding behavior of chironomids seems to prevail in the presence of MPs (in this case PE particles) in sediments. Larvae seem to have no preference between MPs and natural sediments or particulate organic matter (detritus) of similar size (Scherer et al., 2017; Nel et al., 2018; Ziajahromi et al., 2018). Typically, *C. riparius* larvae in their final instar (4^{th} instar) ingest sediment particles in the 60-200 μm fraction as part of their regular diet (Armitage et al., 1995; Ristola et al., 1999; Henriques-Oliveira et al., 2003). However, the size range of the PE particles found in their gut was smaller (average size-range of 40 μm to 60 μm), with very few plastic items reaching 125 μm when exposed to size-class B and C where > 90% of particles were indeed bigger than 63 μm (Figure 1D). The existence of smaller-sized particles in the 4^{th} instar larval gut, compared to the average size of the plastic particles present in the sediment, may be a result of their ingestion and accumulation at early instars. The persistence of PE particles in the larval gut may be due to the lipophilic nature of this polymer which makes them to hetero-aggregate/compact in lipid-rich organs and gut (Paul-Pont et al., 2018). A clear relationship between the amount of ingested MP particles, MP size preference, and the organisms development stage has also been reported for other chironomids and sediment-dwelling annelids (*Tubifex tubifex, Lumbriculus variegatus)* and nematodes (*Caenorhabditis elegans)* (Hurley et al., 2017;

Scherer et al., 2017; Lei et al., 2018; Nel et al., 2018; Redondo-Hasselerharm et al., 2018; Ziajahromi et al., 2018).

Despite their small size, the relatively high content of PE particles (< 63 μm) found along the gut of the *C. riparius* 4th instar larvae underline the low capacity of larvae to eliminate/egest PE microplastics as easily as sediment particles as pointed on previous studies (Scherer et al., 2017). The persistence of PE particles in *C. riparius* larval gut raises, therefore, its potential ecological impact when considering not only life-history traits, such as development and emergence but also suggesting insect larvae as vectors of microplastics to different trophic levels. The present study points that the ingestion and persistence of smaller-sized PE particles in the gut could likely cause a significant reduction in the ingestion of organic items and interfering in food processing (Au et al., 2015) and possibly to affect larval development and imagoes emergence. The larval growth and imagoes emergence revealed to be negatively affected by PE dose and size. However, and considering the size of the ingested particles and the percentage/availability of such particles in the sediment, the observed effects were mainly caused by PE size. Larvae exposed to size-class A encounter higher number of small particles (size < 60 μm, in all tested concentrations) compared with the ones exposed to the size-class B and C, which may explain the higher ingestion and persistence of such small particles in larval gut, and the consequent stronger effects on larval growth and emergence of adults. Naturally, such deleterious effects in larvae exposed to size-class B and C were only observed at higher concentrations, where the encounter with particles < 60 μm size was more likely to occur.

Additionally, the growth impairments at larval stages were translated in terms of emergence delay again with stronger effects being observed for smaller-sized PE class (size-class A), with a delay in females' emergence observed at lower PE concentrations, resulting in a 2-3-fold higher difference in the mean emergence time between females and males. This delay may suggest that growth impairment can be

more severe in females (the ones having a higher delay in EmT_{50}) than in males, at low (and environmental relevant) doses of small-sized MPs. A similar dose- and size-dependent effects of MPs on growth and emergence has also been reported for *Chironomus tepperi* (Ziajahromi et al., 2018), with higher effects for smaller sized MP particles at environmentally relevant concentrations. These results thus suggest negative consequences for the reproduction and population dynamics of midges inhibiting freshwater sediments with microplastics whose long-term effects deserve further study considering multigenerational setups.

The effects of microplastics particles seem to depend not only on features such as polymer size and dose but also on an organism's behavior and ecophysiology. As examples, the ingestion of MP particles (powder or microbeads, PE or PS) with a size range of 12-90 μm proved to affect feeding, growth, and emergence of imagoes, with potential for transference to higher trophic levels (this study, Scherer et al., 2017; Ziajahromi et al., 2017, 2018). On the other hand, the ingestion of MP particles with a size range of 2-12 μm by chironomid larvae appeared not to affect larval growth, imagoes emergence and oviposition, but have been shown to accumulate and be transferred ontogenically into the adult terrestrial life stage (Al-jaibachi et al., 2018; Cuthbert et al., 2019). In addition to the size and dose, microplastics shape might also likely play a role in the effects of microplastics in these organisms. Our study revealed significant effects of irregularly-shaped PE microplastics on chironomids life-history traits at the lowest tested concentration (1.25 g kg^{-1} PE sediment DW). However, to our best knowledge, there is no information on the effects of PE beads within the same (or similar) concentration and size range. Nevertheless, previous studies on marine amphipods pointed for the higher toxicity of irregular shaped MPs when compared to microbeads (Au et al., 2015), which underlines the importance of future studies on his topic.

Although arguable, the concentrations range used in this experiment was set based on recent estimations of primary MPs (size < 300 μm) on freshwaters hotspots (Conkle

et al., 2017). Furthermore, the quantity of such small-sized microplastic particles (< 300 μm) described in field assessment studies seems to be continuously underestimated due to the applied methodologies (e.g. > 300 μm neuston net) (Klein et al., 2015; Conkle et al., 2017; Hurley et al., 2018), and the concentration of small-sized particles are expected to increase in the near future (Rochman et al., 2013). Likewise, contamination by microplastics is not monodisperse (Scherer et al., 2017; Brennholt et al., 2018) and it may reach extremely high concentrations in low river flows (inside of river bends/ curve-deposition) (Hurley et al., 2018), which is the optimal habitat for chironomid larvae and other sediment-dwelling macroinvertebrates such tricoptera, lumbriculidae and lumbricidae.

In this context, the results described here are in line with several others describing deleterious effects of microplastics on aquatic invertebrates and potential for ecosystem-level consequences (Scherer et al., 2017; Ziajahromi et al., 2017, 2018). More research is needed in terms of the effects of these microplastics and their different polymers on crucial physiological processes of freshwater invertebrates, mediating homeostasis such as immune responses and oxidative stress. Moreover, the described effects of MPs on freshwater sediment-dwelling invertebrates might be even serious since weathering and colonization of MPs by microorganisms in freshwaters seems to promote their ingestion by aquatic invertebrates (e.g., Vroom et al., 2017) and increases the molecular interaction with organic contaminants (e.g., Hüffer et al., 2018). Therefore, future studies should consider environmental aged particles combined with POPs, but also to assess effects at lower levels of biological organization (cell and subcellular level).

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Figure 1: Number (1A, 1B and 1C) and size (1D) of Polyethylene (PE) particles ingested by *Chironomus riparius* larvae (DW), after ten days of exposure to three size-class (A: 32- 63 μm, B: 63-250 μm, and C: 125-500 μm). The number of ingested particles was counted in all tested PE concentrations. Such number is presented as mean ± standard error and described with linear regression (*n*=3). For some points, the error bars might be shorter than the height of the symbol. The size of the ingested particles was characterized in the larvae exposed to 1.25 g of PE kg^{-1} dry sediment and are presented as mean (+ symbol) with interquartile range, whiskers, and max/min outliers (*n*=3).

Figure 2: Effects of different size-class: A (32-63 μm); B (63-250 μm); C (125-500 μm), and dose of polyethylene (PE) particles on *Chironomus riparius* larval length (mm) after 10-days exposure. Values are presented as mean ± standard error (*n*=5). Asterisk (*) indicates significant differences ($p<0.05$)

Figure 3: Effects of polyethylene (PE) particles of different size-class: A (32-63 μm); B (63-250 μm); C (125-500 μm), on Chironomus riparius emergence of males and females. Results are expressed as mean emergence time (EmT_{50}) ± standard error, in days (*n*=5). The asterisk (*) denotes significant differences compared to the control treatment (Sprague and Fogels, 1976). For some points, the error bars might be shorter than the height of the symbol. The original concentration-response curves are shown in Figure S1 in the Supplementary data.

Table 1: Optical appearance (magnification: 30×) and particle size distribution analysis of the purchased polyethylene (PE) particles (replicates of 100 g) after vibratory sieve shaking. The particle size distribution was measured through weighting (g) the resulting fractions present in each sieve mesh. Results are presented as mean ± standard error (*n*=2).

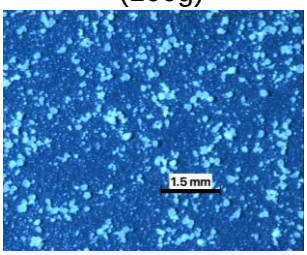
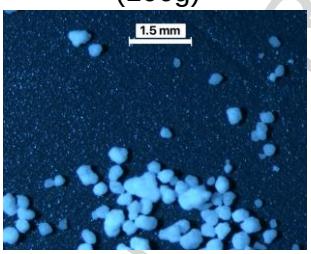
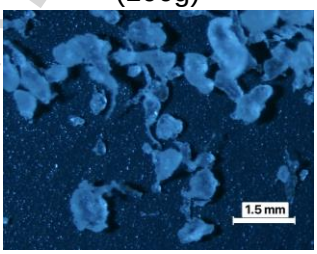
	Average size claimed by the supplier		
	40-48 μm	125 μm	350 μm
	(100g)	(100g)	(100g)
Particle size distribution			
∅ pore (μm)			
≤500	0	0±0	0.06±0.04
>500 - ≤250	0	1.15±0.25	**67.50±7.20**
>250 - ≤125	0	**50.15±2.35**	**26.30±5.70**
>125 - ≤63	0	**39.75±1.75**	5.35±1.05
>63 - ≤32	**94.36±1.36**	8.00±0.70	6.15±5.80
<32	5.54±0.96	0.90±0.10	0.00±0.00
Size-class code	A	B	C

Table 2: Nominal and measured concentration (g kg^{-1} sediment DW) of polyethylene (PE) particles in the sediment, and measured concentration in water column (mg PE per 150 mL ASTM) as result of resuspension process of PE particles, in the beginning of the test (see topic 2.5 in material and methods section). Measurements were performed in the two lowest concentrations of each PE size-class (*n*=3).

	Substrate		
	Sediment		**Water**
PE size-class	Nominal conc. (g kg^{-1} DW)	Measured conc. (g kg^{-1} DW)	Measured conc. (mg/150mL ASTM)
A	1.25	1.23±0.01	0.9±0.3
(32-63 µm)	2.5	2.30±0.01	2.3±0.3
B	1.25	1.22±0.04	1.0±0.2
(63-250 µm)	2.5	2.47±0.02	1.06±0.07
C	1.25	1.18±0.07	0.15±0.07
(125-500 µm)	2.5	2.39±0.03	0.96±0.03

Highlights

- *Chironomus riparius* larvae ingested polyethylene (PE) particles
- Polyethylene particles in the larval gut were mainly in the range of 32-63 µm
- Effects of PE particles on *C. riparius* growth and emergence were dose-dependent
- Emergence was the most sensitive endpoint for female imagoes

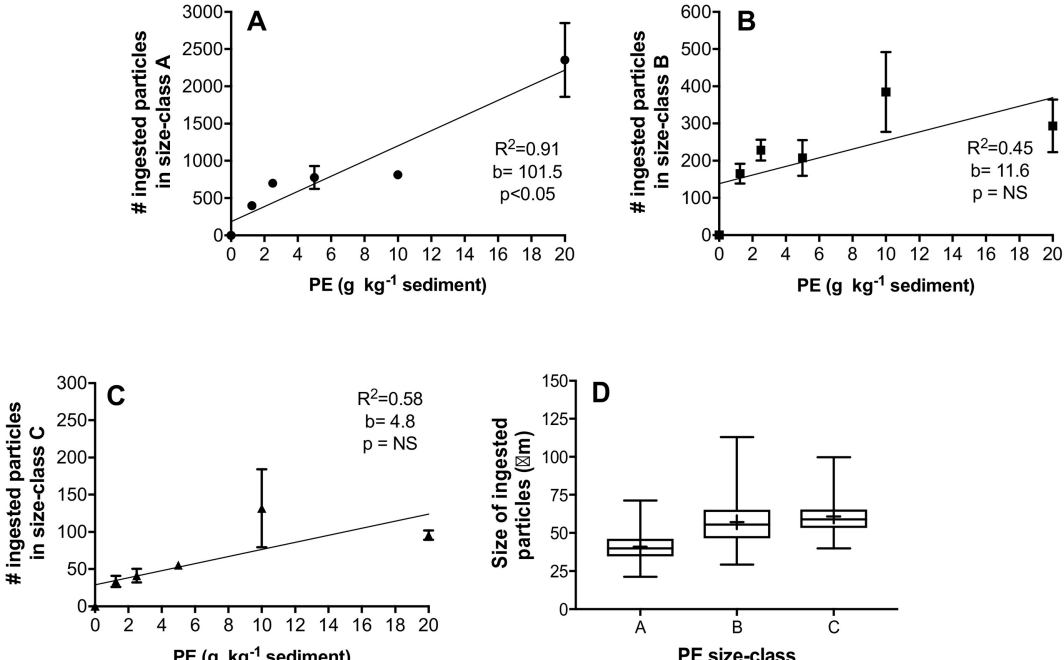

A
ingested particles in size-class A
PE (g kg-1 sediment)
R2=0.91
b= 101.5
p<0.05
B
ingested particles in size-class B
PE (g kg-1 sediment)
R2=0.45
b= 11.6
p = NS
C
ingested particles in size-class C
PE (g kg-1 sediment)
R2=0.58
b= 4.8
p = NS
D
Size of ingested particles (⊠m)
PE size-class
A
B
C

Figure 1

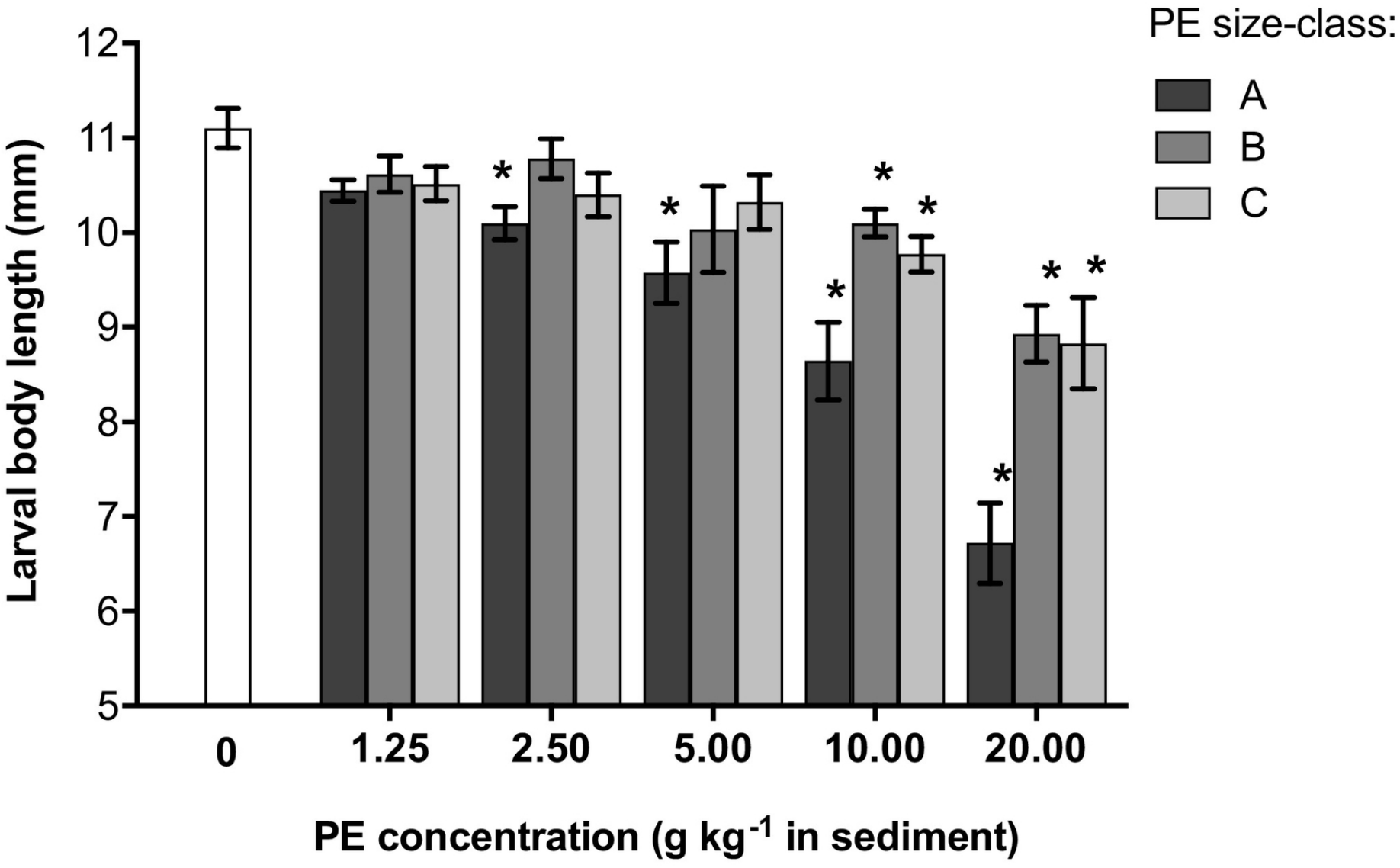

PE size-class:
A
B
C
Larval body length (mm)
12
11
10
9
8
7
6
5
0
1.25
2.50
5.00
10.00
20.00
PE concentration (g kg-1 in sediment)

Figure 2

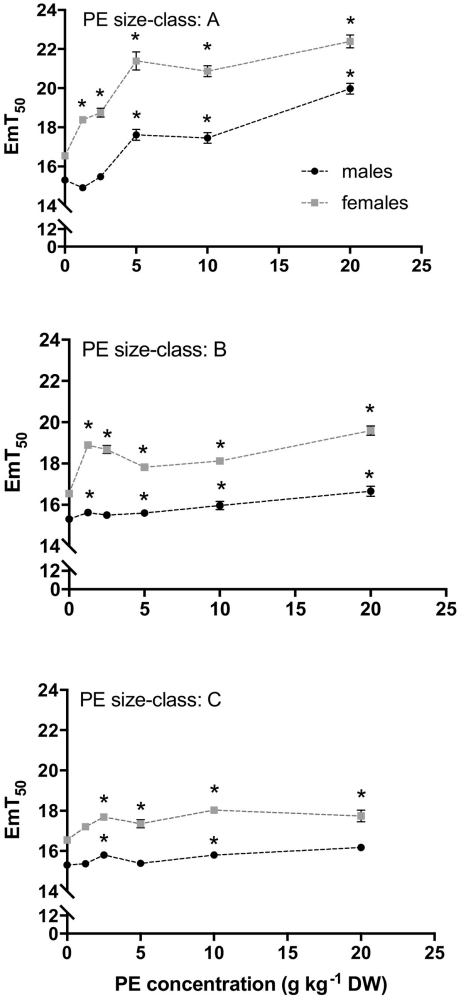

PE size-class: A
PE size-class: B
PE size-class: C
EmT50
males
females
PE concentration (g kg-1 DW)

Figure 3