Polystyrene microplastics ingestion induced behavioral effects to the cladoceran *Daphnia magna*

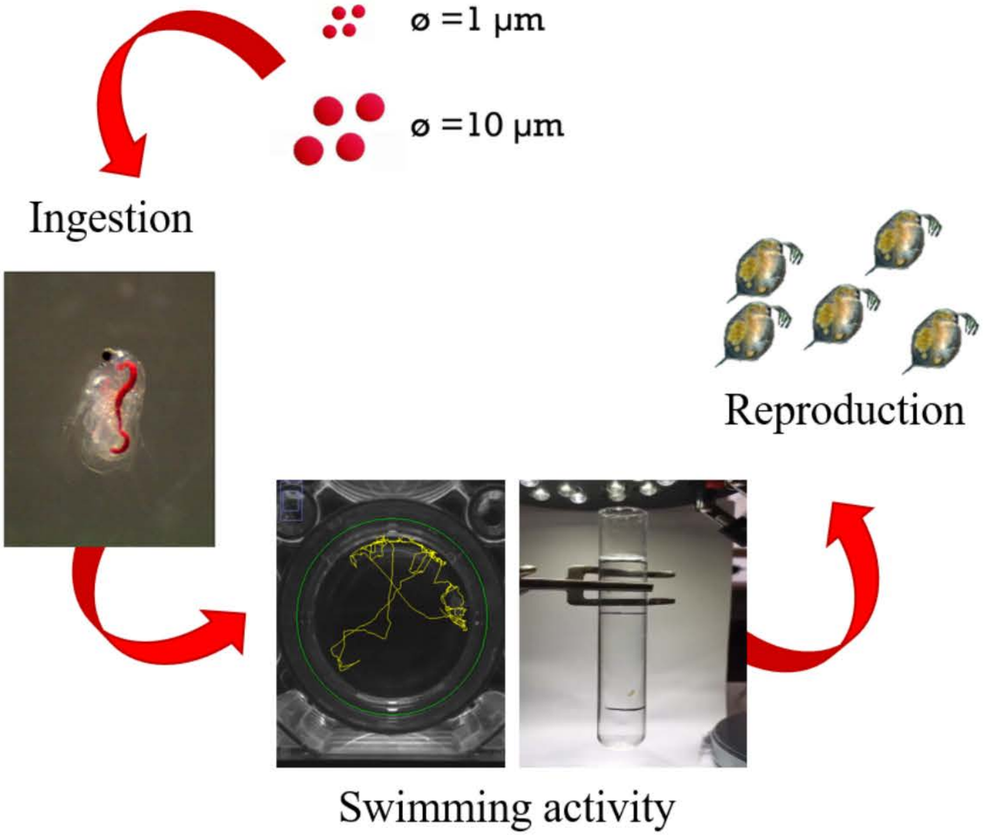
Beatrice De Felice, Valentina Sabatini, Stefano Antenucci, Giacomo Gattoni, Nadia Santo, Renato Bacchetta, Marco Aldo Ortenzi, Marco Parolini

PII: S0045-6535(19)31021-5
DOI: https://doi.org/10.1016/j.chemosphere.2019.05.115
Reference: CHEM 23850

To appear in: *ECSN*

Received Date: 18 January 2019
Revised Date: 5 May 2019
Accepted Date: 14 May 2019

Please cite this article as: De Felice, B., Sabatini, V., Antenucci, S., Gattoni, G., Santo, N., Bacchetta, R., Ortenzi, M.A., Parolini, M., Polystyrene microplastics ingestion induced behavioral effects to the cladoceran *Daphnia magna*, *Chemosphere* (2019), doi: https://doi.org/10.1016/j.chemosphere.2019.05.115.


ø =1 µm
ø =10 µm
Ingestion
Reproduction
Swimming activity

Polystyrene microplastics ingestion induced behavioral effects to the Cladoceran *Daphnia magna*

Beatrice De Felice[a], Valentina Sabatini[b], Stefano Antenucci[b], Giacomo Gattoni[a,c], Nadia Santo[d], Renato Bacchetta[a], Marco Aldo Ortenzi[b], Marco Parolini[a*]

[a] Department of Environmental Science and Policy, University of Milan, via Celoria 26, I-20133 Milan, Italy

[b] Laboratory of Materials and Polymers (LaMPo), Department of Chemistry, University of Milan, via Golgi 19, I-20133, Milan, Italy

[c] Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, United Kingdom

[d] Unitech NOLIMITS, Imaging facility, University of Milan, via Golgi 19, I-20133 Milan, Italy

[*] Correspondence should be addressed to Marco Parolini (marco.parolini@unimi.it)

Abstract

Microplastic (µPs) contamination represents a dramatic environmental problem threatening both aquatic and terrestrial organisms. Although several studies have highlighted the presence of µPs in aquatic environments, the information regarding their toxicity towards organisms is still scant. Moreover, most of the ecotoxicological studies of µPs have focused on marine organisms, largely neglecting the effects on freshwater species. The present study aimed at exploring the effects caused by 21-days exposure to three concentrations (0.125, 1.25 and 12.5 µg/mL) of two differently sized polystyrene microplastics (PµPs; 1 and 10 µm) to the Cladoceran *Daphnia magna*. The ingestion/egestion capability of daphnids (<24 h) and adults was also investigated, as the changes in individual growth and behavior, as changes in swimming activity, phototactic behavior and reproduction. Both particles filled the digestive tract of daphnids and adults within 24 h of exposure at all the tested concentrations. Ingested PµPs remained in the digestive tract even after 96 hours in a clean medium. For both particles, an overall increase in body size of adults was noted at the end of the exposure to the highest tested concentrations, accompanied by a significant increase in swimming activity, in terms of distance moved and swimming velocity, and by an alteration of the phototactic behavior. A significant increase in the mean number of offspring after the exposure to the highest PµPs concentrations of different size was recorded. Polystyrene µPs can affect behavioral traits of *D. magna* leading to potentially harmful consequences on population dynamics of this zooplanktonic species.

Keywords: *Daphnia magna*; behavioral ecotoxicology; microplastics; polystyrene beads; sub-lethal toxicity

1. Introduction

Plastics play a pivotal and irreplaceable role in our society. As plastics are lightweight, durable, inert and corrosion-resistant (PlasticsEurope, 2010) they are extensively used in near inexhaustible applications (Andrady, 2011). However, although the undeniable societal benefits of polymers (Andrady and Neal, 2009), the persistence and the inappropriate disposal of plastic materials have raised the worrisome environmental problem of plastic contamination in both anthropic and natural ecosystems. The presence and the impact of the so-called macroplastics (i.e large plastic debris) have been largely investigated in the marine environment. Such investigations have highlighted that macroplastic contamination represents not only an aesthetic issue with serious economic consequences for tourist and marine-industries, but also a serious threat for the health of marine organisms (Barnes et al., 2009, Sivan, 2011; Cole et al., 2011). In recent years, the attention has been focused on microplastics (µPs), polymer particles smaller than 5 mm that are considered as emerging contaminants of aquatic ecosystems (Avio et al., 2017). Microplastics can be specifically produced to be used in diverse personal care products and as a virgin pellet in different industrial applications (i.e., primary µPS), or can be generated by the break-down of macroplastics (secondary µPs; Eerkes-Medrano et al., 2015). The main sources of µPs are wastewater treatment plants, inland littering after storm and water overflow events, and industrial processes (Brodhagen et al., 2015; Druis et al., 2016). A growing number of studies has highlighted the width of microplastic contamination in marine environment, investigating the presence of different plastic polymers in both abiotic (water and sediments) and biotic (zooplankton, mussels and fish) matrices (Cole et al., 2013). However, to date just a limited number of studies has been focused on microplastic contamination in freshwater ecosystems, although they are recognized as the main source of plastic contamination for marine environment (Wagner et al., 2014; Eerkes-Medrano et al., 2015). In fact, In freshwater environmental matrices, recent studies have estimated a µP contamination as high as 0.001 - 0.1 items/m^2 in lake water, while 0.1 - 1 items/m^2 in rivers. On the contrary, the estimated contamination in sediments was much higher with 10 – 10,000 items/m^2 for lakes and 1 – 1,000 items/m^2 for rivers (Dris et al., 2015). Monitoring surveys of µPs contamination in freshwater ecosystems revealed the presence of diverse plastic polymers, having different environmental fate depending on their physical and chemical features. For instance, polyvinylchloride (PVC) and polyethylene terephthalate (PET) µPs can sink and settle on sediments (Andrady, 2011), while polystyrene (PS), polyethylene (PE) and polypropylene (PP) µPs may float within the water column (Vianello, 2013; Hämer et al., 2014; Li et al., 2016). Microplastics have been reported to interact with aquatic organisms through feeding activity (Cole et al., 2013), dermal uptake (Wagner and Lambert, 2017) or respiration (Watts et al., 2014). A wealth of studies has demonstrated that µPs can efficiently be ingested by 160 marine (Lusher, 2015 and reference therein) and 39 freshwater species (Scherer et al. 2017), including invertebrates, such as cladocerans (Canniff et al., 2018), rotifers (Jeong et al., 2016) and molluscs (Imhof and Laforsch, 2016), and vertebrates, such as amphibians (Hu et al., 2016; De Felice et al., 2018), fish (Lei et al., 2018) and marine mammals (Fossi et al., 2012). A growing number of experimental studies performed on diverse aquatic species has demonstrated that the ingestion of µPs induced diverse sub-lethal adverse effects, including decrease of food uptake (Blarer and Burkhardt-Holm, 2016), onset of oxidative stress (Alomar et al., 2017) and inflammation (Lu et al., 2016), as well as decrease in growth and reproduction rate (Sussarellu et al., 2016; Lo and Chan, 2018). On the contrary, other studies have reported slight or null effects due to µPs ingestion (Hämer et al., 2014; Kaposi et al., 2014; Imhof et al., 2017; Weber et al. 2018). Of particular concern is the exposure and the subsequent effects due to µPs in zooplanktonic filter-feeder species, which indiscriminately ingest µPs during their normal egestion of µPs depend on the particle type, size, and shape (Rosenkranz et al., 2009; Jemec et al., 2016; Frydkjær et al., 2017). Moreover, it has been reported that the ingestion of high concentrations of 1 and 100 µm PE microbeads by daphnids caused a dose and time dependent increase in their immobilization rates (Rehse et al., 2016). It must be considered however, that these results have been obtained by short-term exposures (< 48 h), and that in the natural environment organisms are usually exposed to lower concentrations, but for longer periods. A previous long-term exposure to different concentrations of fluorescent green PE microbeads (63–75 µm) has shown that the ingestion did not affect reproduction in *D. magna*, although the digestive tract resulted filled with µPs (Canniff and Hoang, 2018). In spite of these findings, there still is a dearth of information regarding the potential toxicity of plastic polymers to *D. magna*, mainly on swimming activity and reproduction after long-term exposure. The present study was aimed at exploring the ingestion/egestion and possible effects induced by 1 and 10 µm polystyrene microplastic beads (PSµPs) to this species. Daphnids and adults were exposed to 0.125, 1.25 and 12.5 µg/mL PSµPs for 21 days and ingestion/egestion microscopically investigated at selected times. Moreover, individual growth and behavioural endpoints, including changes in swimming activity, phototactic behaviour and reproduction were also considered. Lastly, we used of Size Exclusion Chromatography (SEC) to detect the possible effects due to permanence in *D. magna* digestive tract on PSµPs molecular weight and distribution index.

2. Materials and Methods

2.1 Daphnia magna husbandry

Daphnia magna used in this work came from a single clone obtained from the Istituto Superiore di Sanità (Roma, Italy). Specimens were reared in a commercial mineral water (San Benedetto® -

beakers at 20.0 ± 0.5 ° C under 16 hours light: 8 hours dark photoperiod, to allow the continuum of the amictic, parthenogenic reproduction (Frey, 1982). Specimens were fed *ad libitum* with a suspension of the unicellular green alga *Pseudokirchneriella subcapitata* (8 x 10^6 cells for individual per day until they were 8-day old, then 16 x 10^6 cells for individual per day) and the yeast *Saccharomyces cerevisiae* (15 x 10^6 cells for mL) three times a week. The culture medium was renewed every second day. Algae were cultured in ISO 892:1989 medium in 2 L flask at 20 ± 2.0 °C under continuous light and shaken through a stirrer. During their exponential growth, algae were left to settle in the dark at 4 °C for a week, then the supernatant was removed and cell density measured under a light microscope by a Burker chamber.

2.2 Polystyrene characterization and stock solution preparation

Red polystyrene microplastics (PSμPs) having two different sizes (Ø = 1 μm and Ø = 10 μm diameter) were purchased from Sigma-Aldrich (Milan, Italy). Polystyrene (PS) was used because it is one of the most abundant plastic type used for food packaging and it is therefore found in freshwaters and marine environments (Li et al., 2016). Moreover, PS has a negligible styrene release in water (Cohen et al., 2002). Chemical-physical properties of the PSμPs beads were provided by the supplier (nominal Ø = 1 μm – calibrated particle diameter = 1.07 ± 0.03 μm, density = 1.51 g/cm^3 and nominal Ø = 10 μm diameter - calibrated particle diameter = 9.86 ± 0.13 μm, density = 1.51 g/cm^3) and confirmed by laboratory analyses. Both commercial standards were chemically characterized by using a Fourier Transformed Infrared Spectroscope (FT-IR) Perkin Elmer Spectrum 100. To confirm the reliability of our exposures, the composition of PSμPs was characterized in the stock solution, obtained by diluting the commercial standard 1:1,000 (v/v) with San Benedetto® mineral water, and in the culture medium used for the experiments, comparing them with a standard food-grade PS used for disposables. Moreover, in order to assess the possible

2.3 Exposure to polystyrene microplastics

The experiment was divided into three steps: *1*) an ingestion (24 hours) and egestion test (96 hours), to investigate if both daphnids (<24-h old) and adults were able to ingest and egest 1 and 10 µm PSµPs; *2*) a 21-day chronic test, to assess the possible effects on reproduction, and *3*) two behavioral assays, to evaluate changes in swimming activity (horizontal swimming) and phototactic behavior (vertical swimming). Each test was performed using PSµPs of both sizes and three different concentrations: 0.125, 1.25 and 12.5 µg/mL. These concentrations were similar to those used in previous studies on other aquatic species, including copepods (Lee et al., 2013), sea urchins and ascidians (Messinetti et al., 2018). All exposures were performed in semi-static conditions, renewing the exposure medium (including control) every single day up to the end of the test, and the beakers were maintained at the same condition as described above. Specimens from all groups were fed every single day until the end of the experiment.

2.4 *Size exclusion chromatography (SEC) analyses*

Both 1 µm and 10 µm PSµPs were analysed using a SEC system consisting of a Waters 1515 Isocratic HPLC pump, four Waters Styragel columns set (HR3-HR4-HR5-HR2) and a UV detector Waters 2487 Detector (λ=255 nm). Analyses were performed at room temperature, using a flow rate of 1 cm^3/min and 40 µL as injection volume. Samples were prepared by dissolving 60 mg of polymer in 1 cm^3 of anhydrous dichloromethane (DCM, anhydrous ≥99.8%).Before the analysis, the solution was filtered with 0.45 µm filters. The analyses were performed in order to check the possible detection of additives such as colorants or others in view of future investigation on the effects of PSµPs egestion on the molecular weight of the polymer. No internal reference was used to avoid possible superimposition of peaks due to species with low hydrodynamic volume.

Light microscopy analyses were performed to assess the capability of both daphnids (<24-hours old) and adults to ingest and egest PSμPs. Fifty specimens were exposed to the three selected concentrations into 100 mL beakers filled with freshly prepared medium together with the opportune amount of PSμPs stock solution to reach the selected concentrations to be tested. A beaker containing the sole culture medium was included as negative control. The ingestion/egestion test was replicated in triplicate. After 1, 3, 5 and 24 h from the beginning of the exposure, five individuals were collected for the microscopy analyses. The remaining specimens were transferred into a clean medium to assess the PSμP egestion (after 1, 3, 5, 24, 48 and 96 h from the beginning of the test). The collected individuals (5 individuals per treatment per each time of analysis, control included) were fixed in paraformaldehyde 4% in 0.1 M phosphate buffered saline (PBS) at pH 7.4. Prior to microscopy analyses, all samples were rinsed in PBS and then examined under a Leica EZ4 D stereomicroscope coupled with an integrated 3 MP digital camera.

2.6 Chronic toxicity test and body growth evaluation

The chronic test was performed according to the standard 21 days chronic reproduction test (OECD, 2004). For each group, control included, 15 replicates (a replicate corresponds to a daphnids less than 24 hours) were performed. The exposures run in 50 mL beakers filled with freshly prepared medium and the opportune amount of PSμP stock solution to reach the concentrations to be tested. Exposure medium was renewed daily and every single day the number of offspring was recorded. At the end of the exposure, the 21-days old individuals were fixed in paraformaldehyde 4% in 0.1 M PBS at pH 7.4. All samples were then photographed at the stereomicroscope and their digital images used for the morphometric analyses by the free Fiji software (Schindelin et al., 2012).

2.7 Swimming activity and phototactic behavior

replicate six times. To assess changes in swimming activity, 30 individuals per treatment were observed at 7, 14 and 21 days of exposure by a video tracking analysis. Videos were recorded with an iPhone 6 by placing organisms in a 12-well plate (11.5 cm x 8 cm x 1.5 cm), called 'arena', filled with 3 mL of water medium (San Benedetto®). After a 30 minutes acclimation, the swimming of each organism was tracked for 30 seconds. The thirty 1080p Full HD videos acquired for each experimental condition were analyzed using the ImageJ plugin Animal Track (Gulyàs et al., 2016). Swimming activity was assessed as the distance moved (expressed in mm) and as the swimming speed (cm/sec). The phototactic behavior test was performed according to Rivetti and coauthors (2016), with slight modifications. After 7, 14 and 21 days of exposure, all samples were individually transferred to the bottom of a glass cylinder (13 x 100 mm with a concave bottom), where they were left 3 minutes for acclimatization in the dark. To avoid interference due to other light sources, the test was performed in a dark room and the bottom of the cylinder was covered by a black cardboard to minimize light reflection. An artificial light was placed above the glass cylinder to mimic a light stimulus. The cylinder was filled with mineral water (San Benedetto®) up to 2 cm from the top (~ 8 mL). After the acclimation period, the light was turned on and the time (in seconds) spent by each sample to reach an arbitrary 'goal line' placed at 6 cm from the bottom of the cylinder was measured. An arbitrary period of 60 seconds was fixed as a temporal endpoint to allow the organism to swim up to the goal line.

2.8 Statistical analysis

The effects of PSμPs exposure on body length, swimming activity, phototactic behavior, as well as reproductive endpoints, of *D. magna* were investigated by using linear mixed models (LMMs), including the treatment and the time of analysis (for body length, swimming activity and phototactic behavior only) as fixed factor, while the identity of the exposure beaker as a random factor to

3. Results

3.1 Characterization of polystyrene microplastics

FT-IR analyses show that both 1 µm and 10 µm PSµPs were very similar to the industrial food grade PS used as a reference standard. Nevertheless, small differences were visible (see Figure 1a,b), especially in the 1,350-1,000 cm^{-1} range. The peaks observed in PSµP were probably due to additives, such as colorants. No significant difference was detected between 1 µm and 10 µm PSµP. The analysis of hydrodynamic volumes of polystyrene used for PSµP is shown in Figure 2a,b: species having higher hydrodynamic volumes have lower retention times than species having lower hydrodynamic volume, therefore they appear earlier in the chromatogram. SEC curves detected the presence of the polymer, at about 40 minutes in 1 µm PSµPs and at about 38 minutes in 10 µm PSµPs, as well as the presence of an additive in 1 µm PSµPs and of two additives in 10 µm PSµPs. Since UV detector was used, such additives are visible because they were UV-sensitive moieties. Therefore, they might be colorants and/or antioxidants or additives used to increase UV resistance of the material. In view of future analyses of egested PSµPs, also these additives must be taken into account, since they are present in the material used even if they are not the polymer itself .

3.2 Effects of 1 µm polystyrene microplastics

No mortality was found over the ingestion (24 h)/egestion (96 h) test in both daphnids and adults. Similarly, no mortality was recorded over the 21-days exposure performed to assess the behavioral effects caused by PSµPs. Microscopy analyses evidenced that 1 µm PSµPs were efficiently ingested by daphnids and adults, both showing their digestive tracts filled with PSµPs already after 1 h of exposure to the highest tested concentration (12.5 µg/L). At 0.125 and 1.25 µg/L, specimens

PSµPs, suggesting that they were unable to purge their gut contents, at least within 96 hours. A marginally non-significant increase in body length in 21-days old individuals ($F_{3,25}$ = 2.322; P = 0.099) was induced by PSµPs exposure (Figure 3a), while significant effects of PSµPs treatment on the swimming activity was found ($F_{3,19}$ = 3.779; P = 0.028). In fact, a significant increase in the distance moved by 21-days old individuals exposed to 1.25 and 12.5 µg/L compared to controls was recorded (Figure 4a). Accordingly, individuals treated with the highest PSµPs concentrations showed a higher speed compared to controls ($F_{3,19}$ = 4.244; P = 0.018; Figure 4c). No significant effect of time × treatment interaction was found in the distance moved and in swimming speed. According to the results related to horizontal swimming, a significant effect of PSµPs exposure on phototactic response was found ($F_{3,19}$ = 9.222; P = 0.001), with individuals exposed to the highest concentration spending more time to reach the top of the cylinder, if compared to control (Figure 5a). A significant time × treatment interaction effect was found ($F_{9,439}$ = 5.141 P < 0.001), with a significant alteration of phototactic response after 7 days of exposure to 1.25 and 12.5 µg/L of PSµPs and after 14 and 21 days only at the highest tested concentration (Figure S2). Lastly, PSµPs treatment induced a significant effect on *D. magna* reproduction, in terms of mean number of offspring ($F_{3,42}$ = 6.258; P = 0.001), but not of number of reproductive cycles ($F_{3,42}$ = 1.402; P = 0.255). In detail, a significant increase in the mean number of offspring was found in individuals exposed to the highest tested concentration if compared to the control group (Figure 6a).

3.3 Effects of 10 µm polystyrene microplastics

According to the experiment performed on 1 µm PSµPs, no mortality of *D. magna* daphnids and adults was found over the ingestion (24 h)/egestion (96 h) test, as well as over the 21-days exposures performed to assess PSµPs-induced behavioral effects. Microscopy analyses showed that 10 µm PSµPs were ingested by daphnids and adults. Already after 1 h of exposure to the highest

(0.125 and 1.25 μg/L). Also for 10 μm PSμPs, a 96 h period in a clean medium was not enough to completely purge the gut content of both daphnids and adults (data not shown). A significant increase in body length of 21-days old individuals ($F_{3,50} = 6.867$; $P = 0.001$) was induced by PSμPs exposure (Figure 3b), being the exposed samples about 5% longer than controls. Overall, a significant increase in swimming activity was noted ($F_{3,19} = 3.656$; $P = 0.030$), with individuals exposed to 1.25 and 12.5 μg/L PSμPs that travelled more distance than controls (4 and 11% independently from the time of exposure, respectively (Figure 4b). Accordingly, a significant increase in swimming speed was recorded in individuals exposed to 1.25 and 12.5 μg/L PSμPs with respect to controls ($F_{3,19} = 4.282$; $P = 0.018$; Figure 4d), independently from the time of exposure. According to the results on horizontal swimming, a significant effect of PSμPs exposure on phototactic response was found ($F_{3,19} = 4.080$; $P = 0.019$). However, in contrast to the results obtained at the end of the exposure to 1 μm PSμPs, treated individuals spent less time to reach the top of the cylinder if compared to controls (Figure 5b), independently from the time of exposure. Lastly, a significant increase in the mean number of offspring was induced by the exposure to the highest PSμPs treatment ($F_{3,48} = 3.561$; $P = 0.021$; Figure 6b), while no significant effect on the number of the reproductive cycles ($F_{3,48} = 2.132$; $P = 0.108$) was found.

4. Discussion

The results of the present study showed that the exposure to 1 and 10 μm PSμP beads were efficiently ingested by *D. magna* and that these particles were able to significantly induced behavioral changes in terms of swimming activity, phototactic behavior and reproduction. Microscopy analyses highlighted the presence of red particles of both sizes in the digestive tract of *D. magna* specimens, including daphnids (<24 h-old), which showed PSμPs already 1 hour after the onset of the exposure at the highest concentration (Figure S1). In all treated groups, daphnids and can be ingested and accumulated in the digestive tract of *D. magna* (Ma et al., 2016; Rist et al., 2017) and of other zooplanktonic species (Cole et al., 2013). Similarly, also PE particles can be ingested by *D. magna* (Rehse et al., 2016): in a 96 h exposure, these authors not only reported that 1 µm PE particles can be ingested by daphnids, but also that this ingestion results in immobilisation of samples exposed to high PE concentrations. Our results comply with our expectations because the size of PSµPs we tested felt in the same size range of those algae *D. magna* usually feed on (1 – 50 µm; Ebert, 2005), confirming that 1 and 10 µm PSµPs that can be found in the water column are available for a filter-feeder organisms, such as *D. magna* and other zooplanktonic species. In contrast, a limited, slow egestion of PSµPs was noted when daphnids or adults were transferred to a clean medium. This finding is in accordance with a previous study on 1-week old *D. magna* specimens in which no significant egestion of 100 nm and 2 µm fluorescent PS beads occurred after 1 hour of ingestion and 24 hours of egestion into a clean medium (Rist et al., 2017). The limited egestion might be due to the absence of food in the clean medium, as the presence of food in the digestive tract is reported to be necessary for the egestion of faeces (Ebert, 2005). Although we did not quantify the ingestion/egestion rate, this hypothesis was supported by the investigation by Rist and coauthors (Rist et al., 2017), who demonstrated that food administration in a plastic-free medium notably affected plastic body burdens, with a decrease of particle mass per individual by 93% and 100% for the 100 nm and 2 µm particles, respectively.

Although PSµPs of both sizes filled up the digestive tract of *D. magna* over the whole experiment, no individuals died over the 21-days exposure period, in all the treatment groups. These results agree with previous studies on different freshwater invertebrate species, in which no mortality was recorded after short-term exposures to µPs at concentrations similar to those tested in the present study (e.g., Imhof et al., 2017; Rist et al., 2017; Weber et al., 2018). Anyway, in spite of no acute several aquatic species, resulting in long-term alterations of physiology, behaviour and fitness of the organisms due to impairments of the energy budget and the whole metabolism (Cole et al., 2015; Wright et al., 2013). However, a previous study on *D. magna* demonstrated that only the exposure to 100 nm and not to 2 µm PS microbeads significantly affected the feeding rate of 1-week old individuals (Rist et al., 2017) likely because of the interaction of nanometric particles with the filter setae and/or the gut wall, thus disturbing the feeding process. Although we did not specifically investigate the feeding rate, considering that the size of particle we tested was in the same dimensional range of algae and similar to that used by Rist et al. (2017), and that the amount of algae administered to our specimens was above the incipient limiting concentration throughout the test, we could suggest that PSµPs exposure did not negatively affect *D. magna* feeding rate (see also Ebert, 2005; Furuhagen et al., 2014). This hypothesis was indirectly supported by the results on the morphology of *D. magna* adults. In fact, 21-days old individuals treated with the highest concentration of both PSµPs were even longer than controls (Figure 3). Indeed, also adults treated with the lowest concentrations of 10 µm PSµPs were significantly longer than controls, suggesting that the increase in the size at the end of the exposures might be due to an enhanced food uptake related to an increased filtering activity and/or to a better efficiency of food absorption in presence of microplastics within the digestive tract. The first hypothesis was supported by our data on swimming activity, which showed that the exposure to PSµPs of both size surprisingly increased the swimming activity of *D. magna*, independently from the individual age. In fact, the exposure to the highest tested concentrations of 1 and 10 µm PSµPS significantly enhanced *D. magna* swimming activity in terms of distance moved and swimming speed (Figure 4). The increase in swimming activity might be explained as an avoidance behavior fulfilled by the organism to swim away from a contaminated environment (Lopes et al., 2004), or as an attempt by the organism to get rid of the

We may speculate that if the organism is able to perceive the presence of particles in the solution, these occluding the digestive tract or hindering the appendage movements, it might increase the swimming activity to look for clean, PSµPs-free water into its carapax, to clean up gut and body cavities or enhance movements of its appendages to rid them of particles. This hypothesis could be particularly true for *D. magna* exposed to 1 µm PSµPs because some particles were found adhering on some external structures of both daphnids and adults, including the carapax and/or the appendages. Interestingly, an increase in phototactic behavior (i.e. vertical swimming activity expressed as the time spent by the individual to travel a vertical path in response to a light stimulus; Figure 5), was also found at the highest concentration of 10 µm PSµPs, while an opposite response was induced by the exposure to 1 µm PSµPs. The discrepancy might be due to the additional weight represented by 1 µm particles on the body and appendages of *D. magna* specimens that lengthened the time due to respond to a light stimulus. In detail, the strongest effect due to 1 µm PSµPs on the phototactic behaviour was noted after 7 days of exposure to 1.25 and 12.5 µg/mL treatments (Figure S2). As *D. magna* started reproduction after 7/8 days from birth, this detrimental effect might be explained as an energy redeployment to the first reproductive event rather than to a light stimulus response. However, this hypothesis needs to be tested in further in-depth experiments. Thus, as the swimming behaviour is strictly related to filter-feeding activity and food uptake is one of the main driving forces of growth and reproduction (Enserink et al., 1993), the enhancement of swimming activity (i.e. horizontal swimming activity) might promote food uptake, body growth and, consequently, reproduction. According to results from body length and swimming activity, an enhanced reproduction, in terms of the mean number of offspring, was induced by the exposure to the highest tested concentrations of 1 and 10 µm PSµPs (Figure 6), while no effects were induced by the exposure to the lowest concentration. Data from the literature reported contradictory results;

increase in the number of neonates from *D. magna* adults exposed to 100 nm PSMPs. In contrast, Besseling et al. (2014) demonstrated in the same species that the exposure to nanopolystyrene (~70 nm) at concentrations up to 103 μg/mL lowered the number of offspring, increased the occurrence of malformation in daphnids and decreased the growth of adults. These findings suggest that the size and the chemical features of the plastic polymers can affect the reproductive success of *D. magna*, although at the same time they indicate that the reproduction of this cladoceran species is rather robust to micro- and nano-plastic stress at and above environmentally relevant particle concentrations (Jemec et al., 2016). The increase in reproductive success might be explained as a terminal effort accomplished by *D. magna* specimens in an adverse, contaminated environment, preferring to invest energy for its fitness rather than to its survival. Although we did not experimentally investigate this hypothesis in the present study, a preliminary long-term experiment showed that *D. magna* specimens exposed to 3 μm PSμPs died before controls (personal communication, unpublished data). Alternatively, we might speculate that individuals exposed to high concentrations of PSμPs increased the efficiency of food absorption, as demonstrated in Pacific oysters exposed to yellow-green fluorescent PS beads (2 and 6 μm diameter; Sussarellu et al., 2016). The enhanced absorption efficiency suggests a compensation to adjust energy intake in response to a possible interference caused by the presence of PSμPs filling the digestive tract of *D. magna* specimens.

5. Conclusion

Results from the present study showed that 1 and 10 μm PSμPs are quickly ingested by *D. magna* daphnids and adults at all the tested concentrations. In contrast to our expectations, the ingestion of PSμPs induced significant enhancements of body length and swimming activity at higher, investigated endpoints, suggesting that PSμP contamination does not seem to pose a worrisome risk for zooplanktonic organisms. Further studies should be planned in order to check for the hypotheses concerning the enhancement of efficient absorption of food and of terminal effort of *D. magna* specimens in very contaminated environments by plastic polymers. Moreover, investigations on the potential effects due to smaller PS spherical particles or to fragments, foams and pellets, which are predominant in freshwater ecosystems, should be necessary to shed light on the impact of PSμPs towards aquatic organisms.

6. 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.

Andrady, A.L., Neal, M.A., 2009. Applications and societal benefits of plastics. Phil. Trans. R. Soc. B. 364, 1977-1984.

Andrady, A.L., 2011. Microplastics in the marine environment. Mar. Poll. Bull. 62, 1596-1605.

Avio, C.G., Gorbi, S., Regoli, F., 2017. Plastics and microplastics in the oceans: From emerging pollutants to emerged threat. Mar. Environ. Res. 128, 2-11.

Barnes, D.K.A., Galgani, F., Thompson, R.C., Barlaz, M., 2009. Accumulation and fragmentation of plastic debris in global environments. Phil. Trans. R. Soc. B. 364, 1958-1998.

Besseling, E., Wang, B., Lüring, M., Koelmans, A.A., 2014. Nanoplastic Affects Growth of S. obliquus and Reproduction of *D. magna*. Env. Sci. Tech. 48, 12336−12343.

Brodhagen, M. Peyron, M., Miles, C., Inglis, D.A., 2015. Biodegradable plastic agricultural mulches and key features of microbial degradation. Appl. Microbiol. Biotechnol. 99, 1039–1056

Browne, M.A., Galloway, T.S., Thompson, C., 2010. Spatial Patterns of Plastic Debris along Estuarine Shorelines. Environ. Sci. Technol. 44, 3404-3409.

Canniff, P.M., Hoang, T.C., 2018. Microplastic ingestion by Daphnia magna and its enhancement on algal growth. Sci. Tot. Env. 633, 500-507.

Cohen, J.T., Carlson, G., Charnley, G., Coggon, D., Delzell, E., Graham, J.D., Greim, H., Krewski, D., Medinsky, M., Monson, R., 2002. Comprehensive evaluation of the potential health risks associated with occupational and environmental exposure to styrene. J. Toxicol. Environ. Health B. 5, 1–263.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., Galloway, T.S., 2013. Microplastic ingestion by zooplankton. Environ. Sci. Technol. 47, 6646–6655.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Galloway, T.S., 2015. The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod *Calanus helgolandicus*. Environ. Sci. Technol. 49, 1130–1137.

Cole, M., Lindeque, P., Halsband, C., Galloway, T.S., 2011. Microplastics as contaminants in the marine environment: A review. Mar. Poll. Bull. 62, 2588-2597.

De Felice, B., Bacchetta, R., Santo, N., Tremolada, P., Parolini, M., 2018. Polystyrene microplastics did not affect body growth and swimming activity in *Xenopus laevis* tadpoles. Env. Sci. Poll. Res. https://doi.org/10.1007/s11356-018-3408-x.

Dris, R.., Gasperi, J.., Tassin, B., 2018. Sources and Fate of Microplastics in Urban Areas: A Focus on Paris Megacity. In: Wagner M., Lambert S. (eds) Freshwater Microplastics. The Handbook of Environmental Chemistry, vol 58. Springer, Cham.

Ebert, D., 2005. Ecology, epidemiology, and evolution of parasitism in Daphnia. Nat. Library of Medicine.

Eerkes-Medrano, D., Thompson R.C., Aldridge, D.C., 2015. Microplastics in freshwater systems: A review of the emerging threats, identification of knowledge gaps and prioritisation of research needs. Water Res. 75, 63-82.

Enserink, L., de la Haye, M., Maas, H., 1993. Reproductive strategy of *Daphnia magna*: implications for chronic toxicity tests. Aquat. Toxicol. 25, 111-123.

Fossi, M.C., Panti, C., Guerranti, C., Coppola, D., Giannetti, M., Marsili, L., Minutoli, R., 2012. Are baleen whales exposed to the threat of microplastic? A case study of the Mediterranean fin whale (*Balaenoptera physalus*). Mar. Pollut. Bull. 64, 2374–2379.

Frey, D.G., 1982. Contrasting strategies of gamogenesis in Northern and Southern populations of Cladocera. Ecology 63, 223-241.

Frydkjaer, C., Iversen, N., Roslev, P., 2017. Ingestion and egestion of microplastics by the Cladoceran *Daphnia magna*: effects of regular and irregular shaped plastic and Sorbed Phenanthrene. Bull. Environ. Contam. Toxicol. 99, 655–661.

Furuhagen, S., Liewenborg, B., Breiholz, M., Gorokhova, E.,2014. Feeding Activity and xenobiotics Modulate Oxidative Status in *Daphnia magna*: Implications for Ecotoxicological Testing. Environ. Sci. Technol. 48, 12886-12892.

Gulyás, M., Bencsik, N., Pusztai, S., Liliom, H., Schlett, K., 2016. Animal tracker: an ImageJ-based tracking API to create a customized behaviour analyser program. Neuroinformatics 14:479–481. https://doi.org/10.1007/s12021-016-9303-z.

Hämer, J., Gutow, L., Köhler, A., Saborowski, R., 2014. Fate of microplastics in the marine isopod Idotea emarginata. Environ. Sci. Technol. 48, 13451–13458.

Hu, L., Su, L., Xue, Y., Mu, J., Xu, J., Shi, H., 2016. Uptake, accumulation and elimination of polystyrene microspheres in tadpoles of *Xenopus tropicalis*. Chemosphere 164, 611–617.

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? PlosOne DOI 12:e0187590. https://doi.org/10.5061/dryad.9d84

Imhof, H.K., Laforsch, C., 2016. Hazardous or not – Are adult and juvenile individuals of *Potamopyrgus antipodarum* affected by non-buoyant microplastic particles? Environ. Poll. 218, 383-391.

Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R., Law, K.L., 2015. Plastic waste inputs from land into the ocean. Science 347, 768–771

Jemec, A., Horvat, P., Kunej, U., Bele, M., Kržan, A., 2016. Uptake and effects of microplastic textile fibers on freshwater crustacean *Daphnia magna*. Environ. Poll. 219, 201-209.

Jeong, C. B., Won, E. J., Kang, H. M., Lee, M. C., Hwang, D. S., Hwang, U. K., Zhou, B., Souissi, S., Lee, S., 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. Science Technol. 50, 8849-8857.

Lee, K., Shim, W.J., Kwon, O., Kang, J., 2013. Size-dependent effects of micro polystyrene particles in the marine copepod Tigriopus japonicus. Environ Sci Technol 47, 11278–11283.

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, 1–8.

Li, W.C., Tse, H.F., Fok, L., 2016. Plastic waste in the marine environment: a review of sources, occurrence and effects. Sci. Total. Environ. 566,333–349.

Lo, H.K.A., Chan, K.Y.K., 2018. Negative effects ofmicroplastic exposure on growth and development of *Crepidula onyx*. Environ. Pollut. 233, 588–595.

Lopes I., Baird, d.J., Ribeiro, R., 2004. Avoidance of copper contamination by field populations of *Daphnia longispina*. Environ. Toxicol. Chem. 23,7.

Lovern, S.B., Strickler, J.R., Klaper, R., 2007. Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C60, and C60HxC70Hx). Environ. Sci. Technol. 41, 4465-4470.

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. Techn. 50, 4054-4060.

Lusher, A., 2015. Microplastics in the marine environment: distribution, interactions and effects. Marine Anthropogenic Litter 245,307.

Ma, Y., Huang, A., Cao, S., Sun, F., Wang, L., Guo, H., Ji, R., 2016. Effects of nanoplastics and microplastics on toxicity, bioaccumulation, and environmental fate of phenanthrene in fresh

Messinetti, S., Mercurio, S., Parolini, M., Sugni, M., Pennati, R., 2018. Effects of polystyrene microplastics on early stages of two marine invertebrates with different feeding strategies. Environ. Pollut. 237,1080–1087.

OECD, 2004. OECD Guideline for Testing of Chemicals (Daphnia sp., Acute Immobilisation Test).

Ogonowski, M., Schür C., Jarenén, A., Gorokhova, E., 2016. The Effects of Natural and Anthropogenic Microparticles on Individual Fitness in *Daphnia magna*. PlosOne DOI:10.1371/journal.pone.0155063

Plastic Europe, Frankfurt, Germany, 2010

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.

Rist, S., Baun, A., Hartmann, N., 2017. Ingestion of micro- and nanoplastics in *Daphnia magna*—quantification of body burdens and assessment of feeding rates and reproduction. Environ. Pollut. 228, 398–407.

Rivetti, C., Campos, B., Barata, C., 2016. Low environmental levels of neuro-active pharmaceuticals alter phototactic behaviour and reproduction in *Daphnia magna*. Aquat. Toxicol. 170, 289-296.

Rosenkranz, P., Chaudhry, Q., Stone, V., Fernandes, T.F., 2009. A comparison of nanoparticle and fine particle uptake by *Daphnia magna*. Environ. Toxicol. Chem. 28,10.

Scherer, C., Brennholt, N., Reifferscheid, G., Wagner, M. 2017. Feeding type and development drive the ingestion of microplastics by freshwater invertebrates. Sci. Rep. 7,17006.

Sivan, A., 2011. New perspectives in plastic biodegradation. Curr. Opin. Biotechnol. 22, 422-426.

Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Pernet, M.E.J., Le Goïc, N., Quillien, V., Mingant, C., Epelboin, Y., Corporeau, C., Guyomarch, J., Robbens, J., Paul-Pont, I., Soudant, P., Huvet, A., 2016. Oyster reproduction is affected by exposure to polystyrene microplastics. Proc. Natl. Acad. Sci. USA 113:2430–2435.

Vianello, A., Boldrin, A., Guerriero, P., Moschino, V., Rella, R., Sturaro, A., Da Ros, L., 2013. Microplastic particles in sediments of Lagoon of Venice, Italy: first observations on occurrence, spatial patterns and identification. Estuar. Coast. Shelf. Sci. 130,54–61.

Wagner, M., Scherer, C., Alvarez-Muñoz, D., Brennholt, N., Bourrain, X., Buchinger, S., Fries, E., Grosbois, C., Klasmeier, J., Marti, T., Rodriguez-Mozaz, S., Urbatzka, R., Vethaak, A.D., Winther-Nielsen, M., Reifferscheid, G., 2014. Microplastics in freshwater ecosystems: what we know and what we need to know. Envrion. Sci. Eu. 24,12.

Wagner, M., Lambert, S., 2017. Freshwater Microplastics. The Handbook of Environmental Chemistry, vol 58. Springer, Cham.

Watts, A.J.R., Lewis, C., Goodhead, R.M., Beckett, S.J., Moger, J., Tyler, C.R., Gallowat, T.S., 2014. Uptake and retention of microplastics by the shore crab *Carcinus maenas*. Environ. Sci. Technol. 48,8823–8830.

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.

Wright, S.L., Thompson, R.C., Galloway, T.S., 2013. The physical impacts of microplastics on

Xu, X.Y., Lee, W.T., Chan, A.K.Y., Lo, H.S., Shin, P.K.S., Cheung, S.G., 2017. Microplastic ingestion reduces energy intake in the clam *Atactodea striata*. Mar. Pollut. Bull. 124,798–802.

Figure captions

Figure 1: panel a) complete FT-IR spectrum of standard PS (red curve), 1 µm and 10 µm PSµP

Figure 2: Size exclusion chromatography **(**SEC) of 1 µm PSµPs (panel a) and 10 µm PSµPs (panel b).

Figure 3: estimated marginal means (±SE) of body length of 21-days old *D. magna* individuals exposed to 1 µm (a) and 10 µm (b) PSµPs. Asterisks above the histograms indicate significant differences compared to the control group (* $P < 0.05$; ** $P < 0.01$).

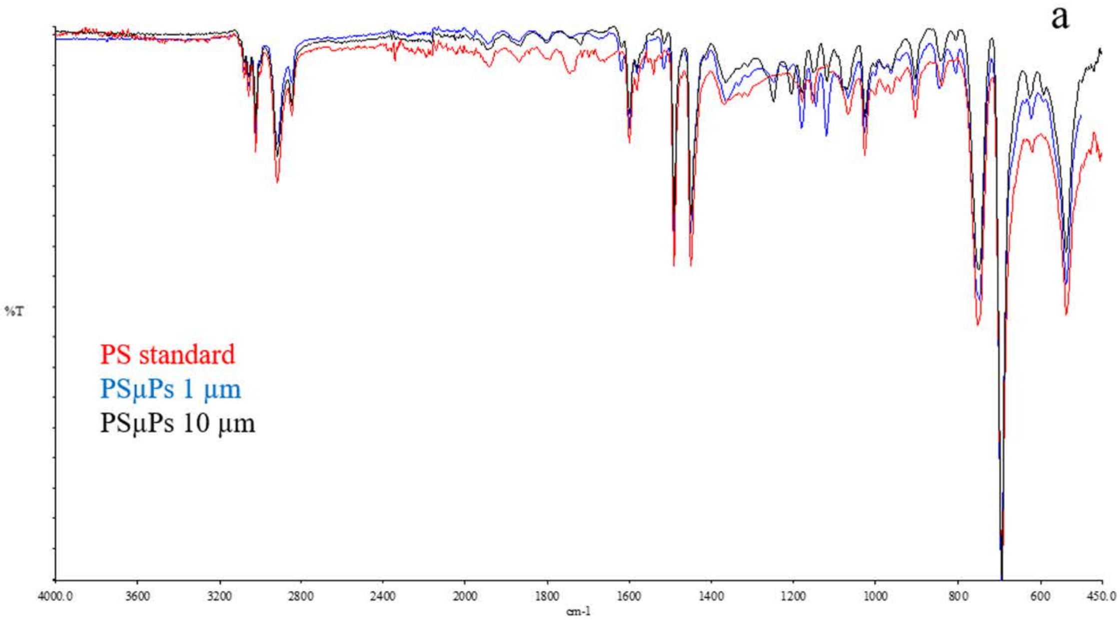
Figure 4: estimated marginal means (±SE) of swimming activity, in terms of distance moved (a and c) and swimming speed (b and d), measured in *D. magna* individuals after the exposure to 1 µm (a-c) and 10 µm (b-d) PSµPs. Asterisks above the histograms indicate significant differences compared to the control group (* $P < 0.05$; ** $P < 0.01$).

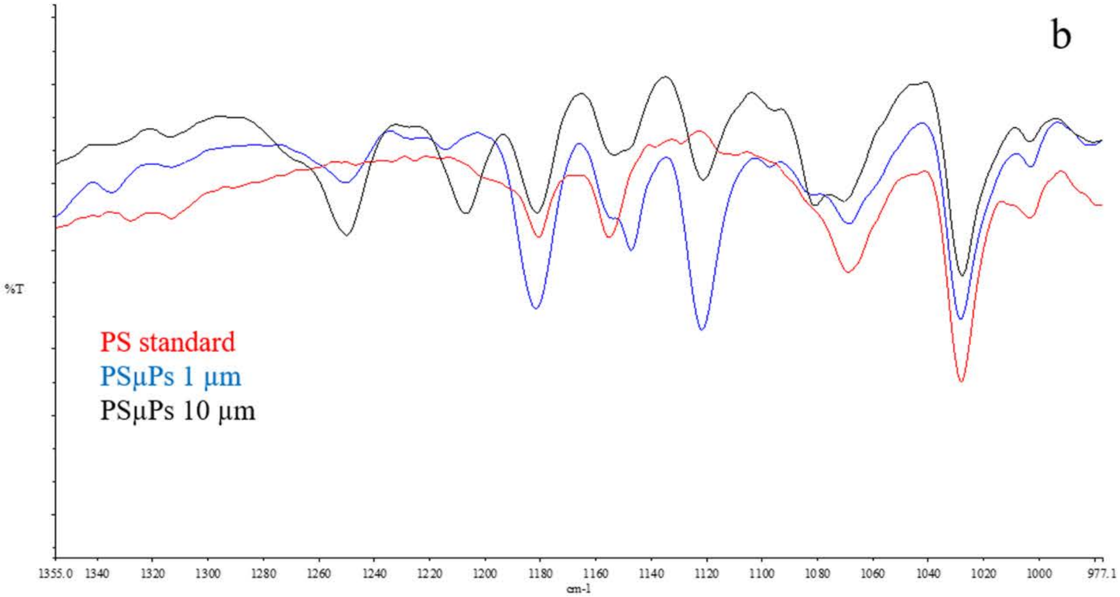
Figure 5: estimated marginal means (±SE) of phototactic behavior measured in *D. magna* individuals exposed to 1 µm (a) and 10 µm (b) PSµPs. Asterisks above the histograms indicate significant differences compared to the control group (* $P < 0.05$; ** $P < 0.01$).

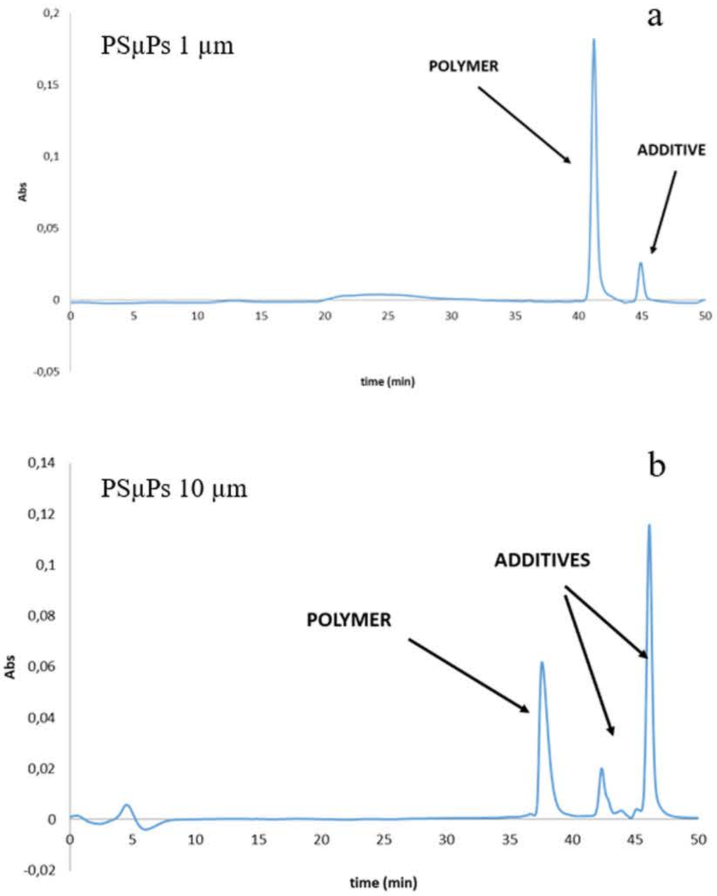
Figure 6: estimated marginal means (±SE) of mean number of offspring generated by *D. magna* individuals exposed to 1 µm (a) and 10 µm (b) PSµPs. Asterisks above the histograms indicate significant differences compared to the control group (** $P < 0.01$).

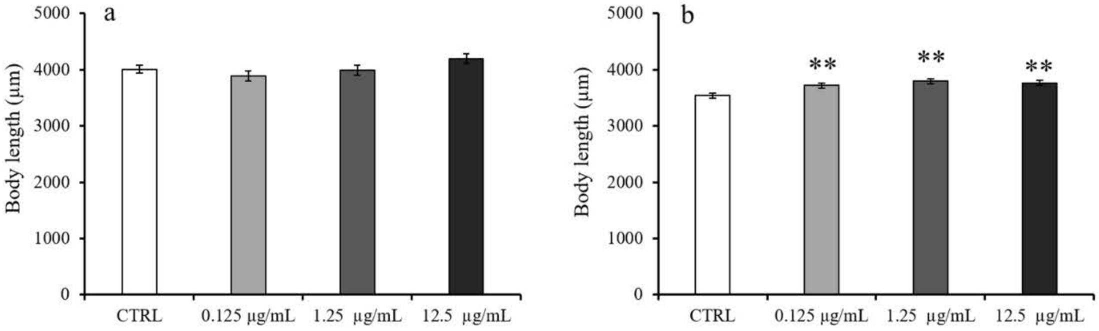
Figure S1: ingestion of PSµPs in daphnids (< 24h; a) and 21-day old (b) of *Daphnia magna.*

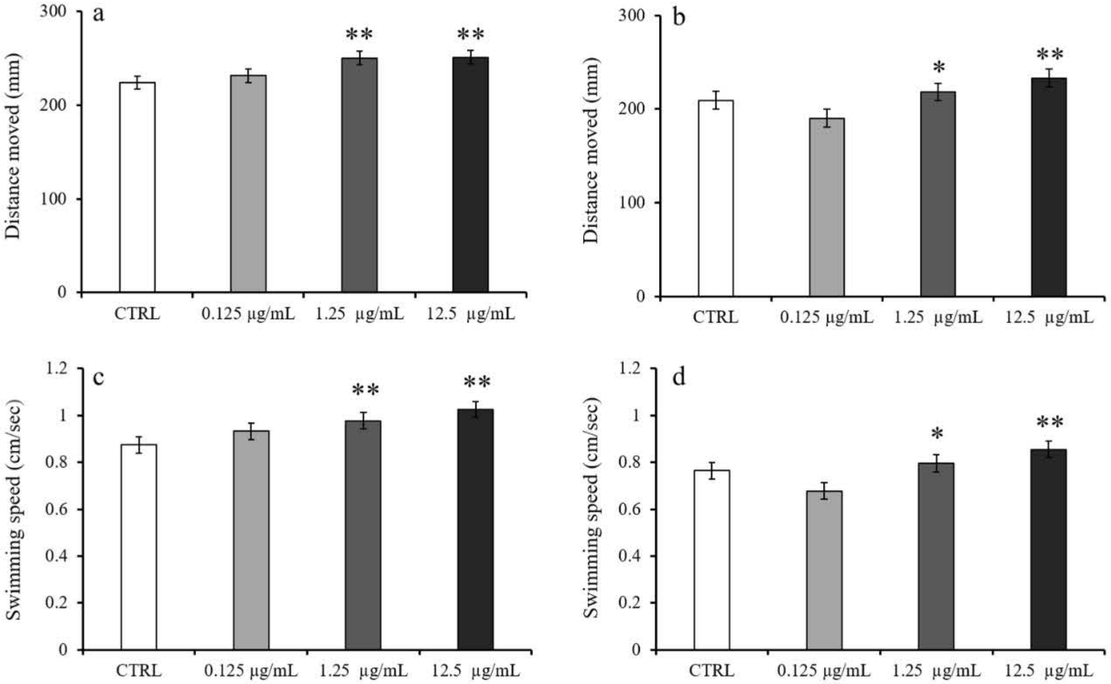
Figure S2: estimated marginal means (±SE) of the time × treatment interaction of phototactic behavior measured in *D. magna* individuals exposed to 1 µm PSµPs. Asterisks above the histograms indicate significant differences compared to the control group (* $P < 0.05$; ** $P < 0.01$).

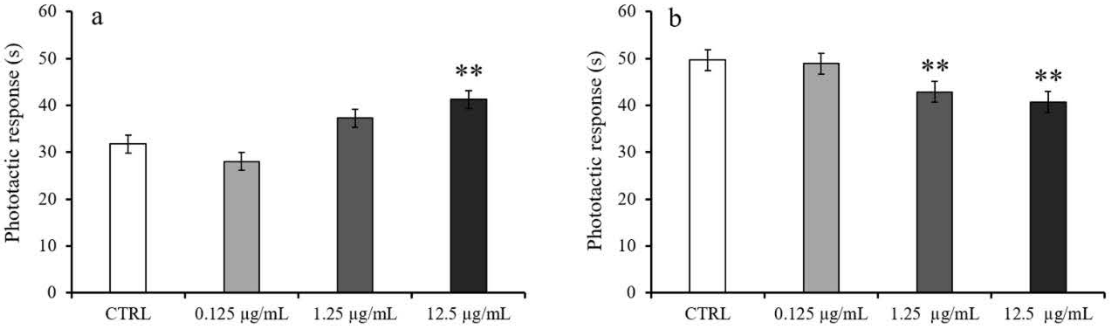

a
%T
PS standard
PSµPs 1 µm
PSµPs 10 µm
4000.0
3600
3200
2800
2400
2000
1800
1600
1400
1200
1000
800
600
450.0
cm-1

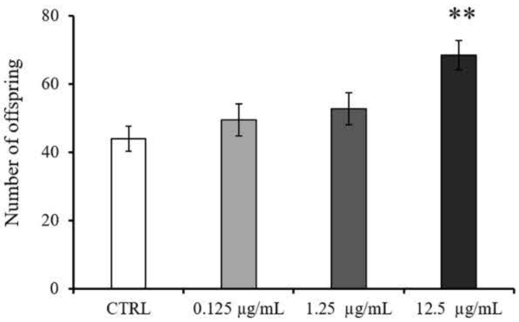

b
%T
PS standard
PSµPs 1 µm
PSµPs 10 µm
1355.0
1340
1320
1300
1280
1260
1240
1220
1200
1180
1160
1140
1120
1100
1080
1060
1040
1020
1000
977.1
cm-1

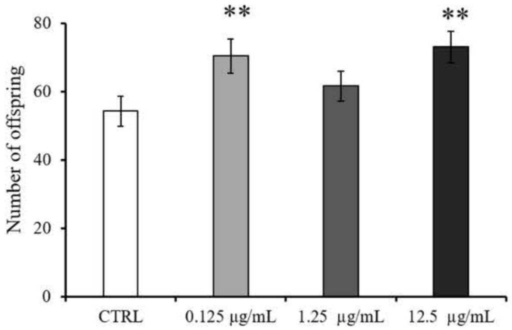

a
PSμPs 1 μm
POLYMER
ADDITIVE
Abs
0,2
0,15
0,1
0,05
0
-0,05
0
5
10
15
20
25
30
35
40
45
50
time (min)
b
PSμPs 10 μm
ADDITIVES
POLYMER
Abs
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
-0,02
0
5
10
15
20
25
30
35
40
45
50
time (min)


a
Body length (µm)
5000
4000
3000
2000
1000
0
CTRL
0.125 µg/mL
1.25 µg/mL
12.5 µg/mL
b
Body length (µm)
5000
4000
3000
2000
1000
0
**
**
**
CTRL
0.125 µg/mL
1.25 µg/mL
12.5 µg/mL


a
Distance moved (mm)
300
200
100
0
CTRL
0.125 µg/mL
1.25 µg/mL
12.5 µg/mL
**
**
b
Distance moved (mm)
300
200
100
0
CTRL
0.125 µg/mL
1.25 µg/mL
12.5 µg/mL
*
**
c
Swimming speed (cm/sec)
1.2
1
0.8
0.6
0.4
0.2
0
CTRL
0.125 µg/mL
1.25 µg/mL
12.5 µg/mL
**
**
d
Swimming speed (cm/sec)
1.2
1
0.8
0.6
0.4
0.2
0
CTRL
0.125 µg/mL
1.25 µg/mL
12.5 µg/mL
*
**


a
60
50
40
30
20
10
0
Phototactic response (s)
**
CTRL
0.125 µg/mL
1.25 µg/mL
12.5 µg/mL
b
60
50
40
30
20
10
0
Phototactic response (s)
**
**
CTRL
0.125 µg/mL
1.25 µg/mL
12.5 µg/mL


**
80
60
40
20
0
Number of offspring
CTRL
0.125 µg/mL
1.25 µg/mL
12.5 µg/mL


**
**
80
60
40
20
0
Number of offspring
CTRL
0.125 µg/mL
1.25 µg/mL
12.5 µg/mL

Highlights

- Behavioural effects caused by polystyrene microplastics on *D. magna* were studied
- Microparticles were observed into the digestive tract of daphnids and adults
- Unexpected increase in body size of adults and swimming activity was noted
- An increase in reproductive effort at high microparticle concentration was noted