Primary and secondary plastic particles exhibit limited acute toxicity but chronic effects on *Daphnia magna*

Elvis Genbo Xu[1,2], Rachel Cheong[1], Lan Liu[3], Laura Hernandez[1], Agil Azimzada[1], Stéphane Bayen[3], Nathalie Tufenkji[1]*

[1]Department of Chemical Engineering, McGill University, Montreal, Quebec, Canada H3A 0C5

[2]Current address: Department of Biology, University of Southern Denmark, Odense, Denmark 5230

[3]Department of Food Science and Agricultural Chemistry, McGill University, Montreal, Quebec, Canada H3A 0C5

*Corresponding author

Corresponding email: nathalie.tufenkji@mcgill.ca

Corresponding address: Department of Chemical Engineering, McGill University, Montreal, Quebec, Canada H3A 0C5

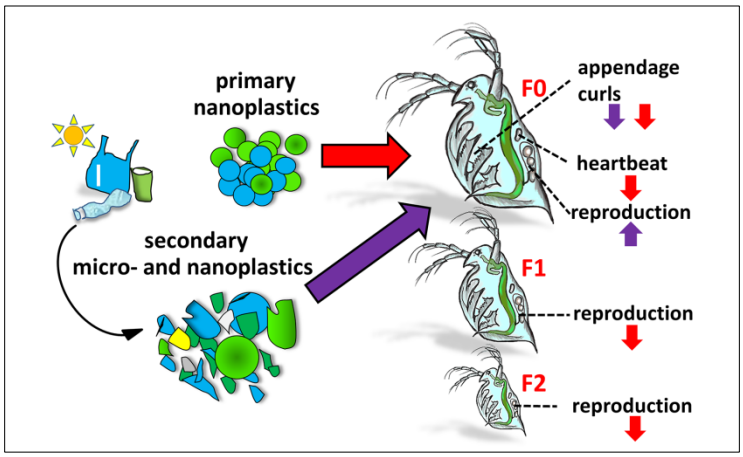
Corresponding Tel.: (514) 398-2999

Fax No.: (514) 398-6678

Abstract

Nanoplastics (NPs; < 0.1 µm), are speculated to be a bigger ecological threat due to the predicted wider distribution, higher concentrations and bioavailability. Primary NPs are manufactured to be that size, while secondary NPs originate from fragmentation of bigger debris. To date, the long-term impact of NPs in freshwater systems, particularly secondary NPs, are not well understood. Thus, we employed a freshwater invertebrate, *Daphnia magna*, to investigate the chronic effects of model primary NPs, fluorescent polystyrene nanospheres (PS-NPs; 20 nm), and water leachate of weathered single-use plastics that contained micro- and nano-sized particles. In experiment 1, parent *Daphnia* (F0) was exposed to 1 and 50 mg/L PS-NPs until the production of the neonates (F1) followed by a two-generation recovery. PS-NPs were mainly detected in the intestine and brood chamber in F0 and transferred to F1 and F2. PS-NPs significantly decreased the appendage curling and heartbeat rate in F0 and reduced reproduction in F2. In experiment 2, the plastic leachate also reduced the appendage curling rate but increased growth and reproduction. The results suggest that the acute toxicity of primary and secondary plastic particles is low even at high concentrations but their chronic and sublethal effects should not be overlooked.

TOC Graphic



Introduction

Over the last few decades, plastic pollution has become a global issue and its negative impacts on the health of ecosystems and human populations are of growing concern. Previous studies have shown that microplastics (MPs; 0.1 µm to 5 mm in size) are widespread in aquatic ecosystems.[1-3] Weathering of a single MP particle can yield millions to billions of nanoplastics (NPs; < 100 nm)[4] and NPs are speculated to be widespread in the environment.[5] To date, however, the environmental concentration of NPs is unknown and little is known about the environmental fate of NPs due to limitations in analytical methods.[6] These small NPs exhibit higher surface-area-to-volume ratios and a higher potential for uptake into cells compared to the bulk plastic material or MPs.[7] It has been shown that NPs can interact with biological molecules which could affect the transport of materials across cell membranes.[8-10] A number of studies have established that NPs can affect the survival, growth, feeding, and reproduction of various organisms.[11] According to their sources, NPs are classified into two general categories. Primary NPs are originally manufactured to be that

size, while secondary NPs originate from fragmentation or breakdown of larger plastic debris. Most existing studies on the toxicity of NPs used well-defined primary NPs, which are very different from nanoplastic fragments (secondary NPs) in the environment with a wide variety of polymer types, shapes, surface characteristics, and particle sizes.[12] Thus, there is a pressing need to address this knowledge gap for better understanding of the potential effects of secondary NPs that may be different from those of primary NPs.

Daphnia magna is an important crustacean in freshwater ecosystems and commonly used for ecotoxicological assays because of its high sensitivity to external stressors, rapid reproduction, parthenogenetic reproductive nature, and ease of maintenance in the laboratory. Bioassays with *D. magna* were also proposed for use as pre-screening of toxicity to mammals.[13,14] Previous studies have documented adverse effects in *D. magna* after MP and NP exposures (e.g., polystyrene nanoplastics, PS-NPs), including immobilization, mortality, abnormal embryonic development, and swimming behavior.[15,16] However, their multigenerational effects and recovery after PS-NP exposure are much less studied.[17] To date, only two studies investigated the multigenerational effects of MPs in daphnids.[18, 19] Moreover, weathering of plastic debris could not only generate secondary MPs and NPs but also release various toxic chemical additives or contaminants, such as plasticizers, flame retardants, metals, stabilizers, antioxidants, biocides.[20] The majority of available toxicological studies focused on assessing the toxicity of plastic particles, but the leaching chemicals can also contribute to the overall toxicity. Such potential indirect toxic effects are less understood than direct ingestion of MPs and NPs.[21,22] So far, only few studies assessed the short-term toxicity of leachate from MPs in sea urchin and mussels.[23,24] Thus, understanding the

long-term combined effects of NPs and plastic-associated chemicals is required to assess the actual environmental risks of NPs.

To fill the knowledge gaps, the present study aims to investigate the long-term effects of both primary and secondary plastic particles using *D. magna* as a freshwater model invertebrate. Our first objective is to reveal whether polystyrene nanospheres (PS-NPs), as the model primary NPs, can be transferred from one generation to the next generation and cause transgenerational effects. The second objective is to assess the chronic effects of real-life plastic leachate from weathered single-use plastics that contains micro- and nano-sized particles as well as organic and inorganic chemicals on the survival, growth, physiology, behavior, and reproduction of *D. magna*. The results of the two experiments together reveal that primary PS-NPs and plastic leachate that contains secondary MPs, NPs, and plastic-associated chemicals are not acutely toxic even at relatively high concentrations but induce different effects in *D. magna* after chronic exposures.

Materials and methods

Culture conditions of Daphnia magna

Daphnia magna were cultured in moderately hard reconstituted water (MHRW) supplemented with 2 µg/L of vitamin B12 (Fisher Scientific, 98%) and anhydrous sodium selenate (Fisher Scientific, >99%) as advised by Environment and Climate Change Canada (SPE1/RM/14, 2000). The colony was maintained at 20 ± 2 °C and a pH of 7.4-7.8 in a photoperiod of 16 h light: 8 h dark cycle under 8W fluorescent 6500K-daylight bulbs (Eiko Global LLC., KS USA). The culture medium was changed daily, and the colony was fed daily with a mixture of *Chlamydomonas*

reinhardtii (concentration ~3.0×10^6 cells/mL) and a Yeast CerophyllMC Trout (YCT) (5 µL/mL; Aquatic Research Organisms Inc., NH, USA).

Polystyrene nanoplastics (PS-NPs)

The fluorescent polystyrene nanospheres (20 nm; ThermoFisher Scientific) were used as model primary NPs. PS-NP stock suspensions were supplied in deionized water containing 2 mM sodium azide. The fluorescent dye used to label the NPs was yellow-green with 505 nm wavelength excitation and 515 nm wavelength emission. The mean diameter of the particles (34.61 ± 0.1 nm) was confirmed using Dynamic Light Scattering (DLS) (Zetasizer NanoZS, Malvern Instruments).

Nanoplastics dialysis and dye leaching experiments

The PS-NP stock suspension (5000 mg/L in 20 mL DI water) was dialyzed in order to remove additives and free dye from the suspension. Dialysis was performed using Spectra7 dialysis tubes (MWCO 1000 Da) in 2 L of deionized water (DI) water which was changed 20 times over 7 consecutive days. The similar size distribution and average size of PS-NPs before and after dialysis were confirmed using DLS (**Fig. S1**). Two passive sampling experiments were used to investigate whether the fluorescent dye could leach or desorb from the PS-NPs. High-purity silicone sheets (127 µm thick, McMaster-Carr, Canada) were cut into a size that was comparable to *Daphnia* (1 mm × 1 mm) using a surgical blade under a light stereomicroscope (Fisher Scientific). The silicone pieces can be used as a passive sampler for fluorescent dye.[25] In the first dye leaching experiment, silicone pieces were placed in the resulting dialysis water 1 h, 2 h, 3 h, 4 h, 18 h, and

2, 4, 7 days after the start of dialysis for a period of 18 h in each case (**Fig. S2a**). In the second dye leaching experiment, silicone pieces in DI water were carefully sealed in dialysis tubes, and then the dialysis bag was placed in 1 mg/L and 50 mg/L dialyzed PS-NPs or 50 mg/L non-dialyzed PS-NPs in 50 mL MHRW for 12 days (**Fig. S2b)**. MHRW medium with both pH 4.5 and pH 7.0 was used to test the potential effect of pH on the release of dye from PS-NPs. The low pH represented the pH in the *Daphnia* intestinal tract.[26] The silicone pieces were imaged at day 12 using a SZX16 fluorescence stereomicroscope (Olympus).

Multigenerational experiment

The bioassays were carried out in a light chamber with a photoperiod of 16 h light: 8 h dark cycle and temperature of 20 ± 2 °C. Parent *Daphnia* (F0) were exposed to 1 mg/L (corresponds to 1.14×10^{13} particles/mL) or 50 mg/L (corresponds to 5.7×10^{14} particles/mL) of 20 nm PS-NPs in MHRW (with algae and YCT) as well as control conditions (MHRW with algae and YCT) from neonate (< 24 h old) until the production of the first neonates (F1). On the last day before hatching (at the embryonic stage 5 of F1),[27] F0 was carefully rinsed 6-8 times with clean MHRW and moved to fresh MHRW to avoid direct PS-NP exposure to F1. *Ex situ* hatching methods, such as open brood chamber to collect F1 using needles, could further reduce the direct PS-NP exposure to F1 but this was not applied in this study to avoid potential stress and damage to both parents and offsprings. The hatched F1 generation was rinsed twice with clean MHRW and then exposed to fresh MHRW for a two-generation recovery period (**Fig. S3**). The 50 mg/L concentration caused no mortality within 48 h according to our previous study.[16] Individual neonates were placed in

separate glass vials each filled with 20 mL of exposure medium. A total of 10 neonates were used per treatment group. The exposure medium was renewed three times a week to maintain the NP concentrations. 200 μL of *C. reinhardtii* and 100 μL of YCT mixture were added after each medium renewal. The pH, oxygen concentration, and temperature of the old and renewed media were monitored to ensure good water quality of the exposure medium.

The mortality and the number of neonates in each vial were recorded daily. Fluorescence images of the *Daphnia* were taken on the first and last days of exposure for each generation using an inverted fluorescence microscope (Olympus IX71, MA, USA). All fluorescence images were obtained at 4× magnification with an exposure time of 1 second. At the end of each generational bioassay, individual *Daphnia* were placed in a 14 mm glass bottom Petri dish (MatTek, MA, U.S.) with a minimum amount (~1 mL) of exposure medium to restrict vertical movement. *Daphnia* was allowed 2 min to acclimatize to the lighting conditions of the stereomicroscope (Fisher Scientific), and then a 1 min video of the swimming was taken using a smartphone mounted onto the microscope eyepiece. Each video was analyzed to obtain heartbeat rate, appendage curling rate, and postabdominal curling rate using Kinovea software (https://www.kinovea.org/). The heartbeat rate was quantified by the number of contractions of the heart viewed per second. The appendage curling rate was defined as a full rotation of the first thoracic leg. The postabdominal curling rate was quantified when the postabdominal claw was brought proximally toward the thoracic appendages. The tentacle beat was measured when the second antennae moved below the helmet and then back.[28]

Preparation of plastic leachate

80.2 g of virgin commercial single-use plastic items composed mainly of water bottles, fruit netting, plastic bags, bubble wrap, plastic spoon, earbuds, and candy wrap (**Table S1**) were cut into small pieces (~ 1 cm × 1 cm) and placed in a clean Erlenmeyer flask containing 4 L of reagent grade water (Ricca Chemical Company, TX USA), resulting in a liquid-to-solid (L/S) ratio of 50 (equivalent to 20 g plastics/L). The Erlenmeyer flask was then covered with a glass lid, sealed with parafilm, and exposed to direct sunlight outdoors for 20 days, after which the contents were filtered using cellulose nitrate filters (pore size 8 μm; Thomas Scientific, Canada) to remove macroparticles and obtain the exposure leachate. It should be noted that the micro and macroparticles larger than 8 μm that account for most of the mass of the total 80.2 g plastic debris were removed from the leachate by filtration. Following the OECD guidelines,[29] the filtrate was converted to MHRW by addition of calcium chloride (Fisher Scientific, Certified ACS Grade), magnesium sulfate (Fisher Scientific, Certified Grade), sodium bicarbonate (Sigma Aldrich, ACS reagent, ≥99.7%) and potassium chloride (Fisher Scientific, BP/EP/FCC/JP/USP Purity grade). This converted filtrate is referred to as 100% plastic leachate.

FTIR and SEM analysis of plastic leachate

The chemical composition of the single-use plastics (**Table S1**) used for leachate preparation was characterized using a Spectrum TWO Fourier Transform Infrared Spectroscopy (FTIR) instrument with a single-bounce diamond (PerkinElmer) in attenuated total reflection (ATR) mode. Each

plastic piece was placed on the FTIR stage, and then the spectrum was collected and compared against the Perkin Elmer-Spectrum-polymer library.

The plastic leachate was imaged using an FEI Inspect F50 scanning electron microscope (SEM). Plastic leachate (1,000 µL) was carefully drop-casted onto a hydrophilic polycarbonate membrane (10 nm pore size) in a clean biosafety cabinet. Only 10 µL of plastic leachate was drop-casted at a time to avoid agglomeration of particles that might bias the size distribution or the quantification of particles. The dried film of plastic leachate was later coated with a 3 nm layer of platinum (Leica Microsystems EM ACE600 Sputter Coater) for SEM imaging. As the particle suspension is polydisperse, imaging was done at two magnifications to capture the broad range of particle sizes. Particles ranging in size from 300 nm to 17 µm and from 10 nm to 800 nm were observed at 2,500× and 100,000× magnification, respectively. Thirty images of the dried film were taken at each magnification. ImageJ analysis software was then used to determine the size distribution and number concentration of the particles. The approximate particle concentrations of the leachate were calculated based on the average number of particles per unit area in SEM images at each magnification and then extrapolated by considering the total area of the dried droplet and the total volume of leachate drop-casted (1,000 µL).

Metal analysis of plastic leachate

Inductively coupled plasma mass spectrometry (ICP-MS) (NexION 300X, Perkin Elmer) was used to determine the metal content (Cr, Cu, Zn, As, Ce, Cd, Al, and Pb) in the plastic leachate. The

quality of the measurements was tested against the blank (Milli-Q water with 2 % v/v HNO_3), QCS-27 standard (High-Purity Standards), Trace Metals in Drinking Water Standard (TMDW, High-Purity Standards) as well as the calibration standards with the observed deviations not exceeding ± 5 %. The details of metal analysis are provided in Supporting Information.

HPLC- Q-TOF-MS analysis and suspect screening of plastic leachate

Plastic leachate samples were analyzed with an Agilent 1290 Infinity II LC system coupled to the 6545 Q-TOF-MS (Agilent Technologies, Santa Clara, USA). Suspect screening was performed with the MassHunter Profiling software series. Data were aligned using Agilent MassHunter Profinder (B.08.00) based on the processing parameters listed in **Table S2**. The statistical comparison of the chemical profiles among the samples was completed using MassHunter Profiler Professional (MPP, version B14.0) using parameters in **Table S3**. The detailed analytical methodology is provided in Supporting Information.

Chronic toxicity tests on plastic leachate

Prior to chronic toxicity tests, an acute toxicity test was performed according to the OECD guidelines[29] at a series of leachate concentrations (100% in MHRW, 50% leachate diluted with MHRW, 10% leachate diluted with MHRW, and MHRW alone as control) over 48 h. Five *Daphnia* neonates younger than 24 h were placed in a glass vial containing 20 mL of the exposure medium. The acute tests were conducted in triplicates. As no mortality was observed in the acute

test, the highest concentration of plastic leachate (100%) was used as the sublethal concentration for a 10-day chronic toxicity test. Day 10 was chosen as the census date here because it provided minimum time to guarantee at least two broods for both control and leachate-treated individuals in the populations. Furthermore, the growth rates of *Daphnia* became stable by Day 10 which enables an effective comparison between the control and leachate-treated *Daphnia*. There were 10 replicates for each treatment group, with each replicate consisting of 1 neonate per glass vial. The control *Daphnia* was kept in MHRW during the experiment. Sublethal endpoints were measured daily in this experiment; namely, swimming, heartbeat rate, thoracic appendage curling rate, and the number of neonates produced per *Daphnia* using the same methods as described above. At the end of the 10-day experiment, SEM imaging was also performed to visualize any micro-sized particles attached on the *Daphnia* body.

Results

Transgenerational effects of PS-NPs

Strong PS-NP fluorescence was detected in the gastrointestinal tract, appendices, and brood chamber in F0 and F1 generations in a concentration-dependent manner. Interestingly, in the 50 mg/L treatment group, PS-NP fluorescence was also detected in F2 generation even after a long two-generation recovery in clean exposure medium (**Fig. 1a**). The survival of F0, F1, and F2 generations was not significantly affected even at high concentration of PS-NP (50 mg/L), and no mortality was observed in the control and 1 mg/L groups over three generations (**Fig. 1b**). There

was no significant difference in the change of body size in all three generations (**Fig. 1c**); however, PS-NP-exposed *Daphnia* reproduced fewer neonates than controls, and the largest difference (~20% fewer neonates than control) was observed in F2 at 50 mg/L exposure (**Fig. 1d**). Furthermore, the swimming behavior was tracked, and no significant difference in swimming distance was detected between treatment groups and controls over three generations (**Fig. 1e**). Exposure to PS-NPs caused a concentration-dependent decrease in heartbeat rate as well as curling rate of appendages in F0, and 50 mg/L PS-NPs significantly reduced the heartbeat rate and curling rate of appendages compared to the control ($p<0.05$; one-way ANOVA; **Fig. 1f, 1g**). The movement of tentacle and postabdominal curls were not affected by PS-NP exposures in all three generations (**Fig. 1h, 1i**).

Characterization of plastic leachate

FTIR measurements indicated that the single-use plastics used for preparation of plastic leachate were composed of high-density polyethylene, polyethylene terephthalate, polypropylene, polyethylene, and a type of nylon (**Fig. 2c, Table S1**). A significant number of micro- and nano-sized particles was detected in the leachate of single-use plastics weathered outdoors under direct sunlight for 20 days. At 2,500× magnification, particles between 300 nm and 17 µm in size were observed, and the concentration of particles in this size range was determined to be $2.12 \pm 0.70 \times 10^7$ particles/mL. At 100,000× magnification, particles between 10 and 800 nm in size were observed, and the concentration of particles in this size range was determined to be $1.08 \pm 0.25 \times 10^{10}$ particles/mL (**Fig. 2a)**. Thus, the weathered plastic leachate contains approximately 500 times more nano-sized particles than micro-sized particles. Several metals, including chromium, copper,

zinc, cerium, and lead were also detected in the plastic leachate (**Fig. 2d**) at concentrations lower than the corresponding LC_{50} values in *Daphnia*.[30] In addition, the plastic leachate samples were also analyzed by HPLC coupled to accurate mass Q-TOF-MS. The total ion chromatograms (TIC) indicated that many chemical compounds were detected in plastic leachates compared to the control water blank (**Fig. 2f**). PCA analysis further indicated a significant difference between blank and leachate samples in both positive and negative ion modes. After applying a fold change > 2 condition, hundreds of molecular features with signals significantly higher in leachate than that in blank were extracted, and tentative molecular formulae were assigned to these features. From the library search, sixteen compounds detected in the leachate were identified as related to the production of plastics with the matching score >70 (**Table S4**). Notably, the identity of bisphenol A (BPA) was confirmed by the mass accuracy (mass measurement error < 0.54 mg/L), isotope fidelity (matching score of 98), and the retention time match using the mass-labeled BPA standard (retention time difference < 0.1 s; **Fig. 2e**).

Chronic toxicity of plastic leachate

The filtered leachate of the 20-day-weathered single-use plastic debris (100%), as well as the diluted leachates (50% and 10%) did not cause any mortality after 24 h and 48 h exposure, suggesting negligible acute toxicity of the plastic leachate. Hence, the undiluted leachate (100%) was used in the subsequent chronic toxicity test. Compared to the controls, plastic leachates induced a statistically significant increase in body length and reproduction ($p<0.05$; t-test; **Fig. 3a-**

d). No effect on the heartbeat rate and swimming distance was evident (**Fig. 3e-h**). Plastic leachate significantly reduced the curling rate of the thoracic appendages ($p<0.05$; t-test; **Fig. 3i, j**), which was consistent with the results of the PS-NP exposure experiment. The 10-day plastic leachate exposure didn't affect *Daphnia* survival, and the observed chronic sublethal effects were possibly due to the co-exposure of secondary micro- and nano-sized particles as well as the leached organic chemicals and metals.

Discussion

To better understand the long-term effects of NPs, we first performed a prolonged exposure to the model primary NPs (PS-NPs) for neonates (F0) until the production of their first offsprings (F1) followed by a two-generation recovery period. Strong PS-NP fluorescence was detected in the gastrointestinal tract, appendices, and brood chamber of F0 and F1 *Daphnia* (**Fig. 1a**). This agrees with previous studies that NPs can pass to eggs and embryos in different aquatic organisms.[17,31-33] Interestingly, for the first time, we observed PS-NP fluorescence in F2 *Daphnia* even after a long two-generation recovery in the clean medium. Our dye leaching experiments showed that a significant amount of fluorescent dye could leach from PS-NPs from the second hour to the fourth day of dialysis (**Fig. S2a**), highlighting the importance of the dialysis purification step prior to *Daphnia* exposure. A similar recommendation was also given by Catarino et al. who showed that dye leached from 500 nm and 1000 nm fluorescently-labeled polystyrene particles.[34] Although a weak fluorescent signal was still detectable on the silicone pieces that were used as a passive dye

sampler after 7 days of dialysis (**Fig. S2b**), the intensity of fluorescence in F0 PS-NP exposed *Daphnia* was much stronger than in the silicone pieces. Given the similar size of *Daphnia* and the silicone piece used, the fluorescence signal (in **Fig. 1a**) contributed by the free dye is expected to be minor, if any, especially in F2 *Daphnia* that were never directly exposed to PS-NPs nor the fluorescent dye. In future studies, for those particularly focusing on uptake and tissue translocation of NPs, dialysis should be used for removal of additives and free dye.

The actual mechanisms of NP transfer through generations remain unclear, but there are several potential routes. Firstly, PS-NPs have been shown to be able to permeate into the lipid membrane, and show high affinity for yolk protein and lipid droplets.[35-37] *Daphnia* eggs and embryos contain rich yolk granules and fat droplets as exclusive energy sources for respiration and embryonic development, and parent *Daphnia* do not secrete nutrients into the brood chamber.[38,39] Yolk granules and lipid droplets are therefore considered as a possible route of NP uptake by *Daphnia* embryos. Appendages and brood chamber-mediated NP-uptake could be another route. Aggregation of PS-NPs was also observed on the caudal appendices, which was suggested as the main route of transfer into the brood chamber, and hence into embryos.[33] It was shown that 25 nm PS-NPs were accumulated within the brood chamber and internalized by embryos when they were still surrounded by a chorion before organogenesis, suggesting the brood chamber as an important embryonic uptake route.[17] Although F1 was not directly exposed to NPs, the brood chamber is open and water flows past the eggs and embryos by the movement of appendages. This implies that the strong NP fluorescence detected in the F1 was likely contributed by multiple exposure

routes as mentioned above. However, it should be noted that PS-NP fluorescence was also detected in F2 that was only and always cultured in clean MHRW, suggesting the transfer of PS-NPs from their parents.

In the natural environment, the detected concentrations of MPs tend to be two to seven orders-of-magnitude lower than the concentrations reported in exposure studies.[40] The actual environmental concentrations of NPs are still unknown due to the limitations of detection methods, but even if the field-measured MP concentrations were extrapolated to the nano-size range used in exposure studies, the experimental concentration of NP is much higher than expected environmental concentrations.[40] It is possible that the estimated environmental concentrations of PS-NPs may have not yet exceeded the concentrations (1 mg/L and 50 mg/L) we tested in the present study. Nevertheless, at some polluted sites, the environmental concentrations of MPs were found to be up to 5.5 mg/L.[41] Therefore, the lower concentration tested in the first PS-NP experiment (1 mg/L) may be considered as environmentally relevant for plastic polluted water, such as urban rivers and lakes, or pollution hotspots. The particle concentrations in plastic leachate were also measured in the second experiment. The leachate particle concentration in size of 10 to 800 nm was determined to be $1.08 \pm 0.25\times10^{10}$ particles/mL, which is 3 orders of magnitude lower than the low concentration of PS-NPs (1 mg/L corresponds to 1.14×10^{13} particles/mL) in the first experiment. Exposure to 1 mg/L PS-NPs did not affect survival rate, physiological endpoints, or swimming behavior, which suggests PS-NP toxicity is not expected at particle concentrations lower than 1 mg/mL. Given that the measured particle concentration in the plastic leachate is much lower than

1 mg/L, it is possible that the observed effects of plastic leachate are associated with the leached chemicals including metals, bisphenol A, and other organic compounds.

The low toxicity of PS-NPs observed contradicts with some previous studies that reported significant acute and chronic toxicity of MPs and NPs at concentrations similar or even lower than those tested in this study.[18,33,42] Such high acute toxicity could be attributed to the toxicity of preservatives such as sodium azide present in the commercial formulations.[16] In this study, significant effects were only observed at 50 mg/L PS-NP exposure, including bradycardia in F0 *Daphnia*. This result is in accordance with the bradycardia observed in zebrafish exposed to PS-NPs,[31,43] but no PS-NP fluorescence was observed in the heart of the exposed *Daphnia*. This could be due to the lower number of PS-NPs present in the heart than in other organs such as intestine, so the fluorescence in the heart might be masked by stronger fluorescence from other organs. Although the sensitivity of fluorescence detection could be enhanced by increasing camera exposure time, a shorter exposure duration (1 second in this study) was necessary to avoid auto-fluorescence of the *Daphnia*. Whether PS-NPs can penetrate and accumulate in the heart of *Daphnia* and how bradycardia is induced by PS-NP exposure thus remains unknown in this study. In addition to bradycardia, the appendage beat rate was also significantly decreased in F0 *Daphnia* exposed to 50 mg/L PS-NPs. This may be due to the attachment of PS-NPs on the appendages of *Daphnia* (**Fig. 2b**). Such reduced appendage beat was also observed in our second chronic experiment where *Daphnia* was exposed to weathered plastic leachate. As *Daphnia* beat appendages to create water flow for filter feeding,[44] the reduced appendage rate may have

implications on the feeding rate especially when food availability is low. It has been proven that *Daphnia* can decrease feeding rate when they are stressed,[45] which may subsequently inhibit reproduction.[46] In a 21-day exposure study, a decreased feeding rate of *Daphnia* was observed after exposure to 100 nm PS-NPs at 1 mg/L.[47] Another study applied smaller PS-NPs (52 nm) and found a reduction in the cumulative numbers of neonates in *Daphnia* caused by PS-NPs.[33] We also observed lower number of neonates in all three generations when F0 *Daphnia* were exposed to 20 nm PS-NPs at 50 mg/L. The largest reduction in the number of neonates in the third generation (F2) is a most concerning finding. Even if NP exposure only occurs during one generation, the population is not able to recover over two generations, and moreover, multiple generations of *Daphnia* are likely continuously exposed to NPs in a real scenario.

Studies assessing toxic effects mediated by direct NP ingestion are accumulating, but the concurrent indirect effects from the release of chemicals associated with plastics are less studied. Most information available reported acute toxicity ($\leq$96 h) of plastic leachate from virgin plastics with high plastic concentrations.[48-50] In contrast, we did not observe acute toxicity of plastic leachate over 48 h. This lack of acute toxicity may be due to the lower plastic concentration, loss of toxic semivolatile and volatile organic additives, as well as the photocatalyzed transformation of toxic additives in the plastic leachate after the prolonged outdoor weathering and leaching processes in the present study. It was previously shown that the 24 h acute toxicity of weathered plastic pellets was lower than that of virgin plastic pellets to the embryonic development of sea urchin, suggesting rapid effects when additives are released from virgin plastics.[23] Additionally,

the survival of *Daphnia* was not affected even after a prolonged 10-day leachate exposure. Interestingly, however, the growth and reproduction of the leachate exposed *Daphnia* were both significantly increased when compared with those of controls. This could be due to the presence of endocrine disrupting compounds such as bisphenol A, which stimulates the growth and reproduction of *Daphnia* at low (or favorable) concentrations.[51-53] The increase in *Daphnia* growth and reproduction may be caused by overcompensation hormesis as an adaptive response to low levels of stress, which can enhance fitness over a finite exposure time.[54] Our chemical analysis revealed a complex mixture of substances released in the plastic leachate including bisphenol A and other organic compounds (**Table S4**). Two recent reviews both conclude that the release and adverse effects of plastic additives are evident and suggest that upcoming research should include plastic additives as a potential hazard.[20,21]

Several important factors were considered to increase the environmental relevance of our toxicity tests. Firstly, the majority of available toxicological studies focused on assessing the acute toxicity of primary model plastic particles, but invertebrates such as *D. magn*a in the natural environment are chronically exposed to MPs and NPs over multiple generations. To our knowledge, the current study is the first to assess the multigenerational toxicity and recovery of primary PS-NPs in *D. magn*a. We further investigated the chronic effects of real-life plastic leachate from weathered single-use plastics that contains secondary MPs, NPs, and plastic-associated chemicals. To prepare the plastic leachate, we used the L/S ratio of 50 (equivalent to 20 g solid plastics/L) that is 2.5 to 25 times lower or more diluted than the standard ratio recommended by European Committee and

American standards for toxicity characterization of solid waste leachate (EN 12457; USEPA Method 1311). Comparing to previous toxicological studies on plastic leachate, our leachate concentration is one of the lowest. For example, Li et al. used 1000 to 5000 cm^2/L, equivalent to 100 to 500 g/L plastics for plastic leachate toxicity test.[55] Lithner et al. used a L/S ratio of 10 (equivalent to 100 g/L) and 4 (equivalent to 250 g/L) for leaching plastic.[48,49] Bejgarn et al. used a L/S ratio of 10 (equivalent to 100 g/L) to prepare plastic leachate for toxicity test.[50] It should be noted that, in the present study, particles larger than 8 μm that account for most of the mass of plastic debris in the leachate were removed by filtration prior to exposure. The particle concentration of smaller particles (10 - 800 nm) in the leachate was determined to be 3 orders of magnitude lower than the low concentration of PS-NPs (1 mg/L; considered as an environmental concentration in contaminated environments such as some urban rivers and lakes). Thus, this measured concentration of particles in the plastic leachate can be considered to be environmentally relevant for cleaner open water bodies. Follow-up studies are recommended to evaluate the plastic weathering process, quantify the release of plastic particles, and assess their toxic effects under more natural conditions, for example, with the presence of environmental microorganisms, dissolved organic matter, and natural particles at lower plastic particle concentrations.

Overall, our results indicate that high concentrations of plastic particles have limited acute toxicity but chronic sublethal effects when the exposure time is prolonged. Understanding differences in toxicity of primary and secondary plastic particles, as well as the contributions of the particles versus the plastic additives, are essential to assessing the actual environmental risks of plastic

particles. This study provides a more comprehensive picture of the chronic effects of primary and secondary plastic particles in *Daphnia*, leading to a better understanding of long-term impacts of plastic pollution.

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Supporting Information Available:

Fig. S1 shows the size distribution of PS-NPs before and after dialysis. Fig. S2 shows the fluorescent dye leaching experiments. Fig. S3 shows the experimental design of the transgenerational experiment. Table S1 lists the plastic items used for plastic leachate preparation and their chemical composition. Tables S2 and S3 describe the data treatment conditions for the suspect screening based on HPLC-Q-TOF-MS data. Table S4 lists the compounds tentatively identified in the plastic leachate.

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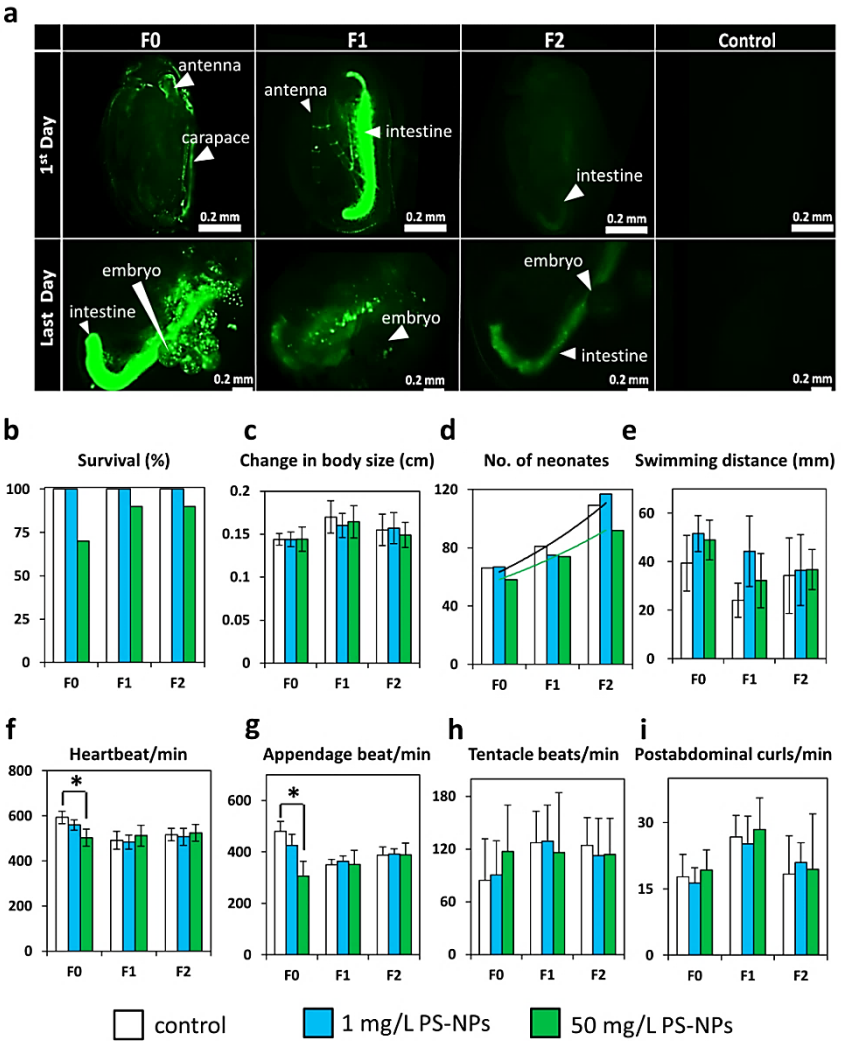

Fig. 1. Transgenerational effects of 20 nm PS-NPs on *Daphnia magna*. (a) F0-F2 *Daphnia* neonates imaged by fluorescence microscopy at the 1st and last day of exposure to 50 mg/L PS-NPs. The white arrows indicate different organs/parts of *D. magna*. The effects of PS-NP exposure on (b) survival, (c) change in body size, (d) number of total neonates reproduced (trend lines are shown as visual aids; the trend lines of control and 1 mg/L are overlapped), (e) swimming distance, (f) heartbeat rate, (g) appendage beat rate, (h) tentacle beat rate, and (i) postabdominal curls at the last day of exposure. An asterisk indicates that the measurement is statistically significantly different from the control [$p < 0.05$; one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test; $n = 10$].

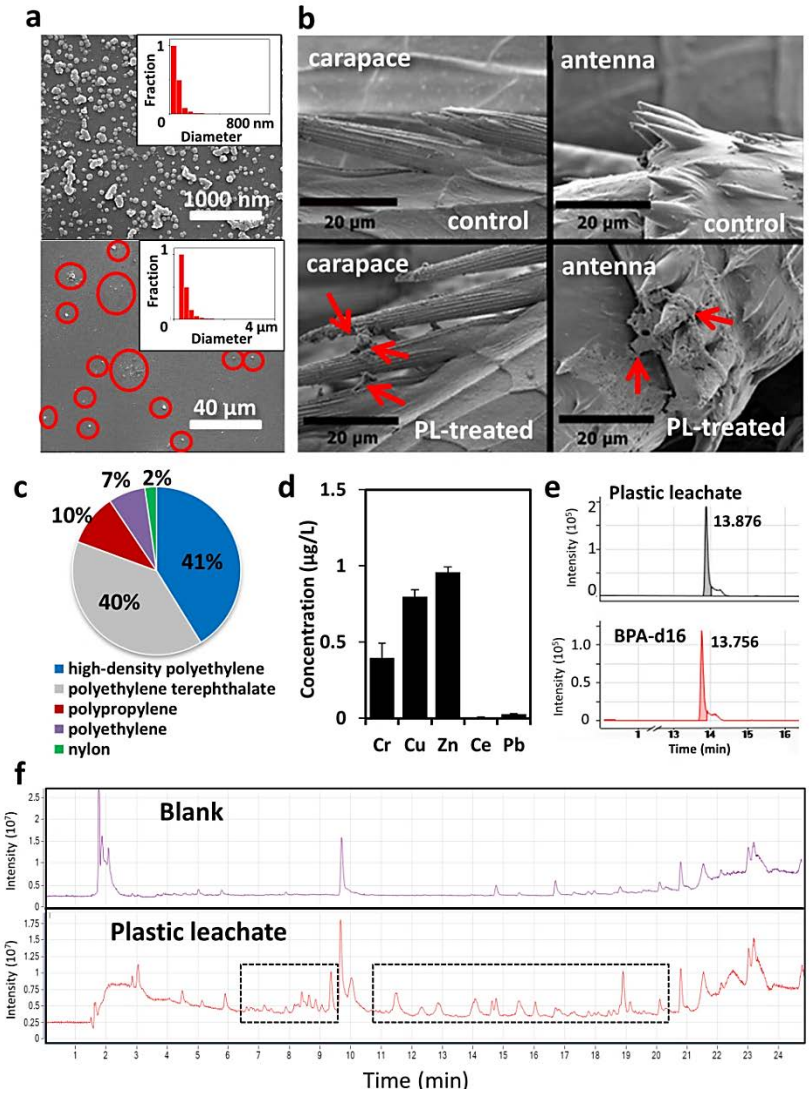

Fig. 2. Characterization of plastic leachate (PL). (a) Representative SEM images of particles in weathered plastic leachate obtained at two different magnifications. The micro-sized particles are highlighted with red circles. The size distributions of particles detected in the leachate are shown in the insets. (b) SEM images of carapace and antenna of control and treated *Daphnia*. Micro-sized particles are indicated with red arrows. (c) The chemical composition of the single-use plastic pieces used for preparing the weathered plastic leachate. The mass-based distribution is shown. (d) The concentration of metals in the plastic leachate. (e) EIC peaks and retention time of BPA-d16 and the corresponding peak in the plastic leachate sample. (f) Comparative chromatograms for HPLC blank and plastic leachate at ESI+ positive mode.

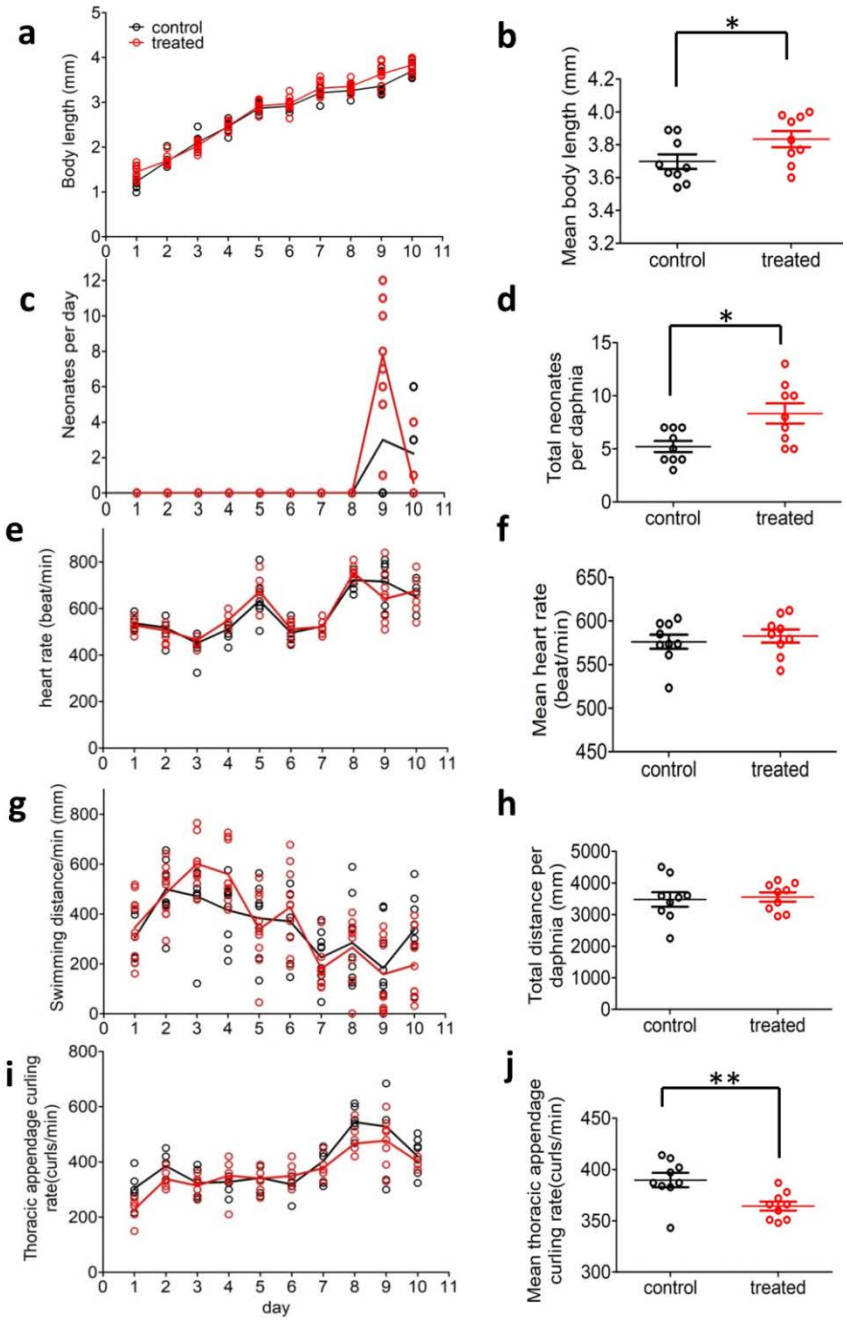

Fig. 3. Responses of *Daphnia magna* to the 10-day exposure of weathered single-use plastic leachate. (a) Body length, (b) mean body length, (c) neonates produced per day, (d) total neonates produced per *Daphnia*, (e) heartbeat rate, (f) overall mean heartbeat rate, (g) swimming distance in one minute, (h) mean accumulated swimming distance per *Daphnia*, (i) thoracic appendage curling rate, (j) mean thoracic appendage curling rate.* p-value < 0.05, ** p-value <0.01; t-test; n = 10.