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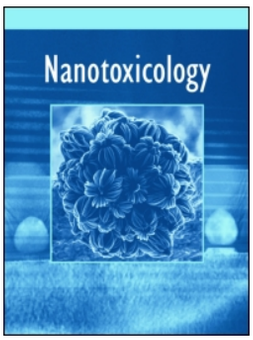

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ORIGINAL ARTICLE

Brood pouch-mediated polystyrene nanoparticle uptake during *Daphnia magna* embryogenesis

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ABSTRACT

Nanoplastic debris is currently expected to be ubiquitously distributed in aquatic environments and an emerging environmental issue affecting organisms across trophic levels. While ingestion of particles receives most attention, other routes of uptake and cellular accumulation remain unexplored. Here, the planktonic filter feeder *Daphnia magna* was used to track routes of uptake and target tissues of polystyrene nanoparticles (PSNPs). A sublethal concentration of 5 mg L^{-1} fluorescent PSNPs (25 nm) was used to monitor accumulation in adult animals as well as their embryos in the open brood pouch. A time series throughout embryonic development within the brood pouch revealed accumulation of PSNP in or on lipophilic cells in the early stages of embryonic development while the embryo is still surrounded by a chorion and before the beginning of organogenesis. In contrast, PSNP particles were neither detected in the gut epithelium nor in lipid droplets in adults. An *ex vivo* exposure of embryos to PSNP demonstrated a similar accumulation of PSNP in or on lipophilic cells, illustrating the likelihood of brood pouch-mediated PSNP uptake by embryos. By demonstrating embryo PSNP uptake via the brood pouch, data presented here give novel insights in bioaccumulation of nanoparticles and likely other lipophilic contaminants. Since this uptake route can occur within a diverse array of aquatic organisms, this study warrants consideration of brood pouch-mediated accumulation in efforts studying the hazards and risks of nanoparticle contamination.

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KEYWORDS
Lipid droplets; nanoplastic; fluorescent nanoparticle; crustacean; embryonic accumulation

Introduction

Increasing amounts of small plastic debris build up in natural systems mainly due to the gradual breakdown of larger plastics such as disposable packaging materials by weathering and photodegradation (Ivar Do Sul and Costa 2014; Bouwmeester, Hollman, and Peters 2015). The contamination of microplastics (< 1 um) in aquatic ecosystems is detected worldwide, even in remote areas (Hammer, Kraak, and Parsons 2012; Cozar et al. 2014; Eerkes-Medrano, Thompson, and Aldridge 2015), since most plastics are not biodegradable and remain in suspension due to lower density than water. Laboratory experiments suggest these particles can further degrade to nanoplastics (< 100 nm; Lambert and Wagner 2016). The small sizes of the plastic particulates make them susceptible to ingestion or cellular accumulation by biota. Their presence is, therefore, posing an increasing threat to our ecosystems and the organisms living therein. However, routes of nanoplastic ingestion and cellular accumulation receive little scientific attention (da Costa et al. 2016).

Studies to date have demonstrated that even though planktonic organisms such as *Daphnia magna* mainly filter out particles with sizes ranging from 0.1–5.0 µm (Gophen and Geller 1984), nanoparticles (NPs) are readily ingested as they adsorb onto algal cells (Nolte et al. 2017). Although larger NPs appear to pass the gut and are rarely taken up (Mendonça et al. 2011; Adam et al. 2015), smaller sized NPs (10–40 nm) such as ZnO NPs, polystyrene NPs (PSNPs) and nanowires have been demonstrated to pass the gut epithelial membrane (Santo et al. 2014; Rosenkranz et al. 2009; Mattsson et al. 2016). Our current notion of cellular targets of PS particles relies on

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their lipophilic property causing them to accumulate in or on lipid droplets that are scattered along the digestive tract of daphnids (Rosenkranz et al. 2009). These lipid droplets are dense aggregates of lipids that can become very large and contain various organelles (e.g. ribosomes and mitochondria) that allow for the synthesis of the egg-yolk precursor vitellogenin. This is subsequently transported via the hemolymph to the ovaries to form yolk granules (Goulden and Hornig 1980). Already in the earliest stages of *Daphnia* embryogenesis, these yolk granules and bigger fat droplets become visible (Giardini, Yan, and Heyland 2015), and serve as exclusive energy source during development as egg-carrying *Daphnia* do not secrete nutrients into the brood pouch (Green 1965). Thus, yolk granules are currently considered as a potential route for maternal transfer and exposure of daphnid embryo's (entailing all developmental stages until release from brood pouch) to polystyrene particles.

However, embryos are also in direct contact with the ambient water since the brood chamber is open to provide a continuous stream of water entering at the abdominal end, passing the eggs or embryos, respectively, while flowing to the anterior end of the chamber where it moves into the ventral carapace chamber. This stream of water is driven by the movement of the thoracic limbs and ensures provisioning of eggs and embryos with oxygen (Seidl, Pirow, and Paul 2002). It is, therefore, conceivable that the introduction of NPs within a brood pouch due to ventilation results in direct contact of embryos to suspended NPs (Rosenkranz et al. 2009), suggesting that brood pouch-mediated NP-uptake could be an important uptake route for many aquatic organisms that rely on this mode of reproduction. However, to date, it remains uncertain whether an actively ventilated brood pouch can indeed serve as a route of NP uptake.

The purpose of this study was to investigate whether and at what stage plastic particles in the nano-range are taken up by *Daphnia magna* embryos during development from egg to embryo, and ultimately to newly born daphnids (called neonates). The experimental set up was designed to elucidate the major uptake routes and sites, visualizing accumulation of fluorescently labeled PSNPs in developing *Daphnia magna* in the brood pouch and *ex vivo*, as well as maternal uptake and transfer in laboratory incubations.

Materials and methods

NP characterization

PSNPs (nominal size 25 nm) were purchased from ThermoFisher Scientific (Waltham, U.S.). These particles are internally dyed with Firefli^{TM} Fluorescent Green (468/508 nm) to prevent dye leaching into aqueous media. A number of techniques were applied to characterize the PSNP in the *Daphnia* culturing medium Elendt M4 (Samel et al. 1999). The shape and size were assessed by transmission electron microscopy (TEM; JEOL 1010; JEOL Ltd., Tokyo, Japan) after 1 h of incubation in exposure medium. Size distribution of hydrodynamic diameter and surface zeta potential measurements were performed in exposure medium without addition of algae at 0 h and after 3 days using a Zetasizer Nano-ZS instrument (Malvern Instruments Ltd, Malvern, UK). Automatic measurements were performed in triplicates in each of the three replicates. Since particles were designed to prevent dye leaching into aqueous media, dye dissolution was not characterized.

The settling of the PSNPs in Elendt M4 exposure medium with and without algae containing 5 mg L^{-1} PSNP was analyzed by measuring fluorescence in the supernatant. Of each sample, the top one-quarter of the total volume was taken as supernatant and its fluorescence was measured in a glass cuvette with a Steady State Fluorescence Spectrometer (Edinburgh Instruments Ltd, Livingston, UK). The excitation wavelength was set at 468 nm and emission wavelength from 478 to 550, according to the manufacturer's leaflet. Medium controls with and without algae were included. The samples were measured at 0 h, 0.5 h, 1.5 h, 2.5 h, 4.75 h, 23 h, 29.75 h, and 52 h. The bottles remained unmoved throughout the entire experiment. A five-point calibration curve was obtained by measuring variable amounts of PSNP ranging from 0.04 to 25 mg L^{-1} at 508 nm and plotted against counts, yielding a linear calibration curve (Figure S1, Supplementary Information, SI). This calibration curve together with the assumption that data was linear over the wavelength range considered in this study was subsequently used to express PSNP concentrations as mg L^{-1}.

Culturing of Daphnia magna

The freshwater crustacean *Daphnia magna* from the Leiden University stock was reared in Elendt M4 medium in a climate chamber at 18 degrees Celsius under a constant light-dark cycle (14:10). The Elendt M4 medium was renewed twice weekly and aerated for 24 h before use. Daphnids were fed three times a week with a *Pseudokirchneriella subcapitata* monoculture. To keep a constant feeding density, *P. subcapitata* cells were collected by centrifugation and resuspended in a volume of Elendt M4 medium to give an algal concentration of 4×10^7 million cells per mL measured photospectrometrically at 684 nm. The final concentration in all cultures was 5×10^5 cells per mL.

Exposure of egg-carrying Daphnia magna *to PSNP*

Adult daphnids that just released a clutch of embryos and thus had an empty brood pouch were selected from the mass culture to assess PSNP uptake in embryos. Control animals ($n = 8$) were put in glass beakers containing 39.5 ml medium supplemented with 0.5 ml algae of the standardized concentration. The medium of exposure animals ($n = 8$) was additionally supplemented with 5 mg L^{-1} PSNP or 5.825×10^{11} particles mL^{-1}, respectively. In a pilot experiment, this PSNP exposure concentration was determined not to affect mortality or reproduction in a 21-day chronic exposure ($n = 10$, data not shown). Green-fluorescent PSNPs were sonicated for 15 s using an ultrasonic water bath (USC200T, VWR, Amsterdam, The Netherlands) before adding it to the medium. All media were refreshed every three to four days. Incubations were not aerated during exposure since oxygen saturation remained well above 90% during these short-term incubations as revealed by preliminary incubations. Four embryos were exposed throughout embryogenesis until day 3 after release from the brood pouch and to assess retention time of fluorescent particles in embryos, four embryos were exposed until pigmented eyes in embryos became evident and monitored until day 3 after release from the brood pouch. For each treatment, 4 control animals were taken along and treated in the same way except for the addition of PSNP. Egg-carrying *Daphnia* were imaged using fluorescence microscopy. Embryos were staged according to Kast-Hutcheson, Rider, and LeBlanc (2001). Briefly, in stage 1 the embryo's shape is spherical with no evidence of cellular differentiation; in stage 2 cellular organization becomes evident and the outer egg membrane (chorion) is shed off; in stage 3 the head capsule and second antennae have differentiated; in stage 4 the pigmented eye is evident; in stage 5 the second antennae are extended and the inner vitelline membrane ruptures; and stage 6 represents the fully developed neonate after release from the brood pouch.

Ex vivo *exposure of* Daphnia magna *embryos to PSNP*

To assess the potential for direct PSNP accumulation in embryos within the brood pouch, an *ex vivo* experiment was performed. Adult *Daphnia* that deposited eggs into the brood pouch within the past 24 h were chosen from the mass culture for the experiment. Embryos were removed from the brood pouch by applying gentle pressure on the posterior part of the brood pouch using forceps and the developmental stage 1 was confirmed using a stereomicroscope. Staged embryos were randomly distributed in a 48-well plate and filled with 2 mL of aerated Elendt M4 ($n = 24$) or medium containing 5 mg L^{-1} PSNP ($n = 24$). *Ex vivo* embryos were incubated under the same conditions as adults (described above) and examined with the confocal microscope every 12 h until the neonatal stage was reached. Mortality and developmental abnormalities were noted.

Imaging techniques

Daphnia embryogenesis within the brood pouch was imaged by fluorescence microscopy using a Leica MZ 16FA equipped with a digital camera (DFC 420) and image acquisition software of Leica. GFP filter (excitation at 470/40 nm, barrier at 525/50 nm) exposure was set at 2.5 s, gain at 2.0 and gamma at 0.6. Transmission settings were set at exposure 146.4 ms, gain 1.0 and gamma 0.6. All animals, control and exposed, were pictured using the same settings throughout the experiment. To identify lipid droplets, the *Daphnia* embryos were stained with the lipophilic dye Nile red (Sigma-Aldrich, St. Louis, U.S.) according to Jordão et al. (2015). The Nile red

stock solution was prepared in acetone at a concentration of 60 μM. *Daphnia* embryos were gently flushed from the brood pouch and incubated in 1.5 μM Nile red in Elendt M4 medium for 30 min in the dark, rinsed in medium for 1 min to remove excess dye followed by imaging using the red fluorescence (DsRed) filter (excitation at 545/30 nm, barrier at 620/60 nm) exposure set at 448.9 ms, gain at 2.0 and gamma at 0.6.

Confocal microscopy was performed on a Zeiss LSM (Zeiss GmbH, Germany) using an Argon ion laser with an excitation wavelength of 488 and an EC Plan-Neofluor 10x/0.30M27 objective, an EC Plan-Neofluor 20x/o.50 M27 objective and a C-Apochromat 63x/1.20 W Korr UV-VIS-IR M27 glycerol immersion objective. Green fluorescent PSNPs were excited at 488 nm and the fluorescence emission was collected with a 505–530 band pass (BP) filter. Confocal imaging was performed for detailed imaging of embryos from *ex vivo* exposure, adult gut epithelium and algal cells. Embryos from the *ex vivo* exposure were imaged alive using the 20x objective, a laser gain of 557 and variable pinhole dependent on the stage of the embryo: 225 (stage 1), 355 (stage 2), 551 (stage 3), 355 μm (stage 4). Stage 5 embryos were imaged using the 10x objective and a laser gain of 557 and a pinhole of 551 μm. In order to image the gut epithelium within the objective's working distance, daphnids were crushed between two glass slides. The 20x objective, pinhole setting of 137 μm and laser gain of 771 were applied. Algal cells were imaged using a 63x objective with the pinhole set to 111 μm and the laser gain to 657. All the laser settings were established in pilot experiments to be able to distinguish between autofluorescence and fluorescence caused by PSNP particles. Limit of detection of fluorescence is dependent on the technique used (fluorescence or confocal microscopy), settings, tissue density, and amount of fluorescent particles accumulated in the tissue.

Results

Characterization of the PSNP

The initial size of about 25 nm is verified by TEM imaging. However, larger aggregates were formed immediately after PSNPs were suspended in Elendt M4 medium (Figure 1(a)). This was confirmed by size distribution analysis using DLS measurements showing an average size of PSNP aggregates of 159 nm at 0 h. Particle agglomeration increased over a time span of 4 days (Figure 1(b)). The zetapotential was on average −11.8 mV at the beginning of the exposure and did not significantly change throughout the exposure period (Figure 1(c)). The fluorescence of the exposure medium (supernatant measured in triplicates) at different time points up to 52 h is shown in Figure 1(d). The results show that PSNPs were suspended in the exposure medium, and some settling of the particles occurred over time. At the peak wavelength of 508 nm, the initial measured concentration (5.28 mg L^{-1}) was reduced by 63% to 3.34 mg L^{-1} after 52 h. However, the signal was still clearly above the background signal of medium with algae. Since the background signal of only algae in Elendt M4 remained in the same range, the impact of algal growth on the suspension of the PSNPs appeared minimal. Therefore, medium renewal every 3 to 4 days seems sufficient for a constant exposure. Furthermore, no difference in fluorescence in medium with algae and without was detected (data not shown).

NP adherence to algal cells and uptake by adult Daphnia

PSNPs adsorbed to the exposed daphnids (Figure S2) and to the outer surface of algae cells (Figure S3). Uptake of algae with PSNPs by daphnids leads to highly fluorescent gut content. However, the fluorescent PSNPs were not seen to cross the intestinal epithelium nor were seen to be accumulated in lipid droplets surrounding the gut after a short-term exposure of 2 days (Figure 2). A few fluorescent spots were found in the epithelium of the gut, however, due to the sickle shape, these seem to be whole algal cells and thus artifacts originating from the crushing procedure rather than showing a pattern of PSNP uptake. From this result, it was concluded that maternal PSNP uptake was not the primary route of particle transfer to embryos.

Identification of PSNP uptake during embryogenesis

Adult *Daphnia* were exposed from the moment that their brood pouch was empty. Within the first hour, fluorescent particles were visible in the gut. Fluorescence started to become visible in the eggs

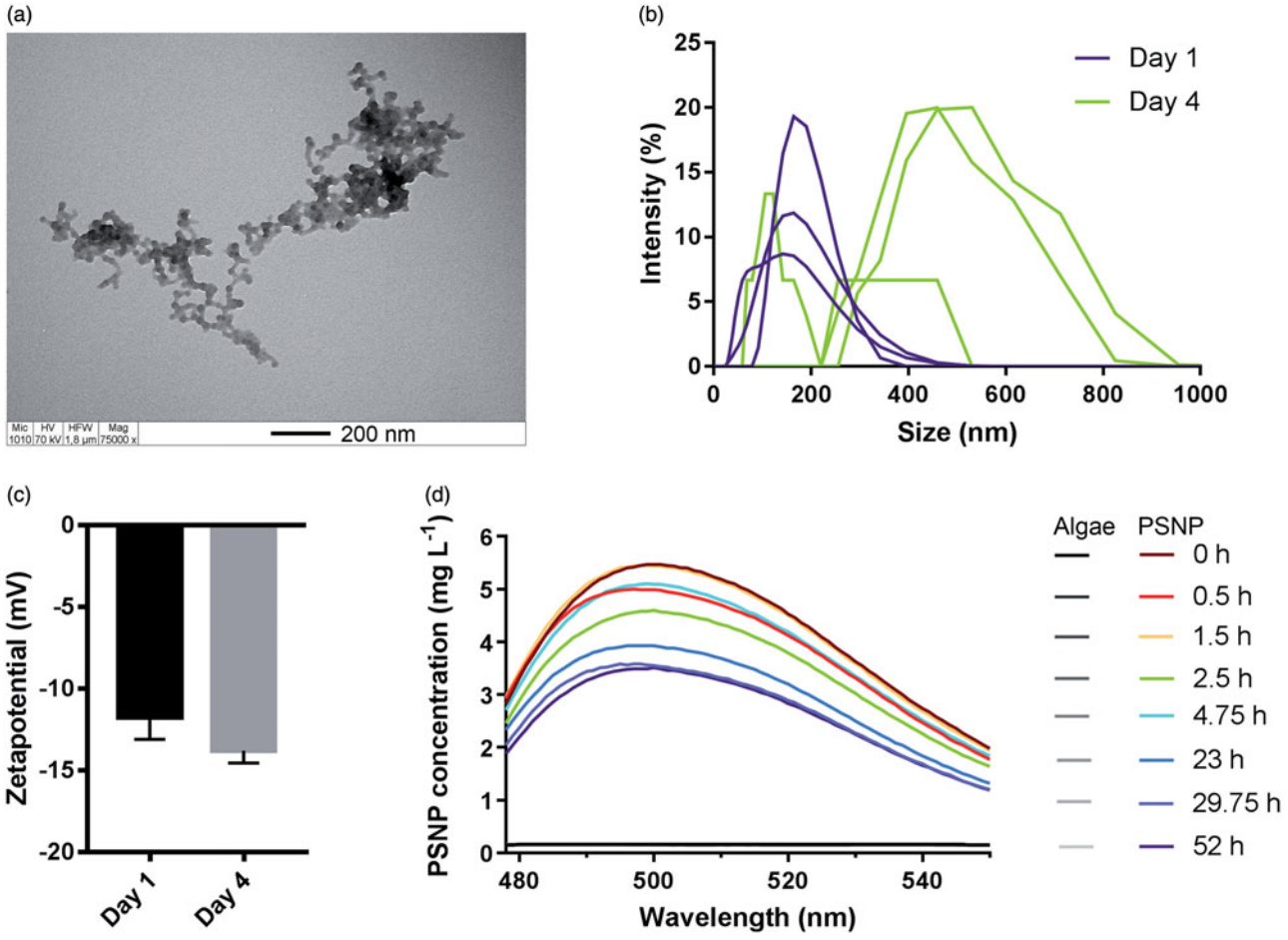

Figure 1. Characterization of 25 nm green fluorescent polystyrene nanoparticles (PSNP) in Elendt M4 medium. (a) TEM image after 1 h in solution. Scale bar: 200 nm. (b) size distribution of the hydrodynamic diameter measured by DLS, (c) zeta potential ($n = 3$), (d) settling of PSNP in medium containing algae over time (colored lines) and background of medium containing algae only (gray lines) measured by a fluorometer, expressed as mg L^{-1} as approximated by a linear calibration (Figure S1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

within the brood pouch at embryonic development stage 3, that is after hatching from the outer chorion and when yolk granules and fat droplets of different sizes become distinguishable features in the embryos. In developmental stage 4, fluorescence is strongly visible in the dorsal region of the embryo (Figure 3). Of the eight clutches we followed through embryogenesis, all showed distinct fluorescence in comparison to the control.

The fluorescent structures were suspected to be polystyrene particles inside or on the fat droplets. To confirm that the structures are fat droplets, newly born neonates were stained with the lipophilic dye Nile red. After the staining, the suspected droplets were fluorescent red when imaged with a fluorescence microscope (Figure 4), thus this is confirming that the droplets where PSNP accumulate are fat droplets.

The embryos and neonates kept the PSNP fluorescent fat droplets even when the PSNP exposure was stopped at stage 4, that is when the pigmented eye was evident. 2 h after exposure was terminated, the fluorescence was barely detectable anymore in the gut of the mother, whereas the embryos retained the PSNP in their lipid droplets (Figure S4). The neonates remained fluorescent until about 6 h after birth, indicating that the particles can be excreted by the cells or detach from the cell surface. When the PSNP exposure was continued after stage 4 and beyond birth, the droplets remained fluorescent until they disappeared altogether after about 2 days. By this time the neonate has used up the yolk.

The *ex vivo* exposure of embryos from stage 1 to 5 allowed detailed monitoring of fluorescence appearance with higher detection limit. While the

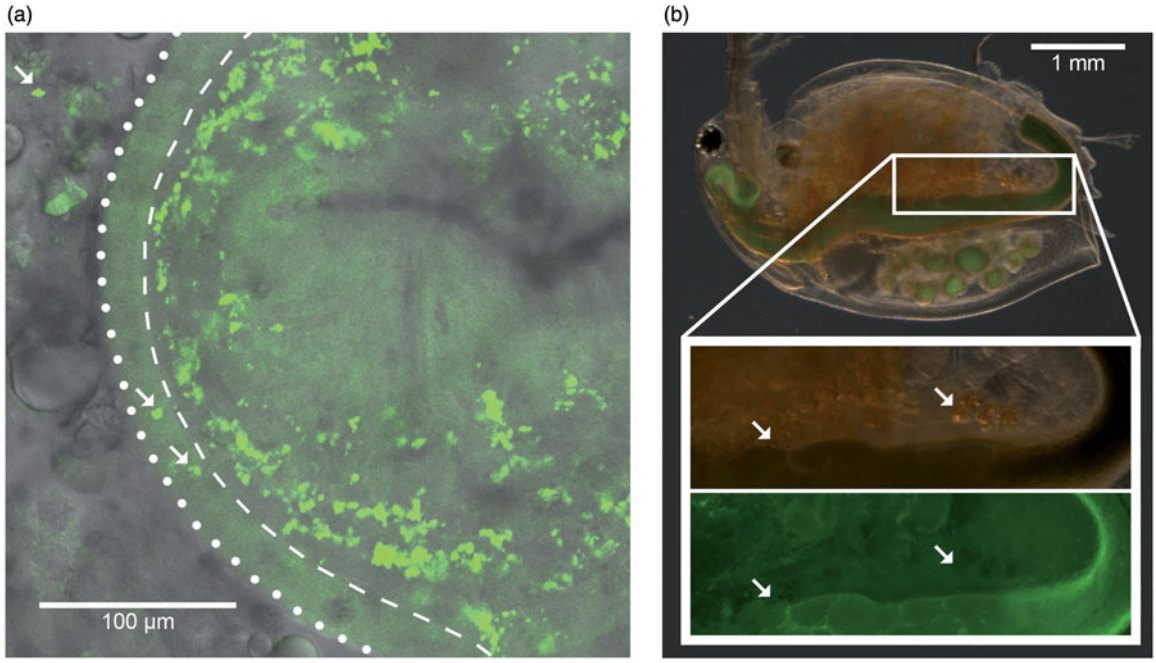

Figure 2. Representative image of PSNP in the intestine and lipid droplets of an adult *Daphnia magna* exposed to PSNP for 2 days ($n = 8$). (a) The PSNPs (green) are present inside of the gut but did not cross the epithelial border structurally. The intestinal epithelium is between the basement membrane (dotted line) and the apical membrane (dashed line). Fluorescent spots outside of the gut (arrows) are likely a result of sample preparation (crushing). (b) Accumulation of PSNP in lipid droplets (arrows) of adult animals was not detected using a GFP filter exposure duration of 2.5 s, gain of 2.0 and gamma of 0.6. The exposure duration may be too low to detect PSNP in maternal lipid droplets, however a short exposure duration was necessary to avoid autofluorescence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

brood pouch exposure only allowed to capture a fluorescence signal from stage 3 onwards, the *ex vivo* exposure revealed uptake of PSNP as early as stage 2 (Figure 5). At this stage, the embryos are surrounded by a two-layered chorion, which seems to be permeable for NPs. All of the exposed *ex vivo* embryos exhibited the same pattern of fluorescence until neonatal stage while all controls remained non-fluorescent. No increased mortality or malformation was observed in comparison to the control.

Discussion

The results presented in this study demonstrate a currently overlooked uptake mechanisms in which *Daphnia* embryos accumulate PSNPs from the surrounding water in the brood pouch. These embryos do not feed actively yet, suggesting absorption to the body epithelium. The *ex vivo* exposure experiment performed in this study indeed confirms that daphnid embryos can accumulate PSNPs from their surrounding water. Even though we did not find clear indications for accumulation of PSNPs in maternal lipids, the PSNPs might still be present in maternal tissue but not be detected due to stringent microscopy settings to avoid autofluorescence. Embryos are suspected to have a higher tissue density and abundance of lipids and thus the PSNP fluorescent signal is detected there first. In fact, PSNPs have been observed to accumulate in maternal lipid droplets (Rosenkranz et al. 2009), suggesting that adults are exposed equally and that daphnid embryos in natural contaminated environments may accumulate PSNPs via multiple uptake routes. Although the relative importance of potential routes of PSNP accumulation in nature remains the subject for further investigation, our data clearly suggest that exposure of *Daphnia* embryos to nanoplastics from the surrounding water or test medium can be a relevant route for uptake. This route of uptake was observed here for a lipophilic PSNP, and therefore might be as well important for other lipophilic NPs, for instance, fullerenes (Fang et al. 2007). Furthermore, lipophilic organic compounds such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), brominated flame retardants and organophosphate pesticides may target lipid droplets during *Daphnia* embryonic development, although these

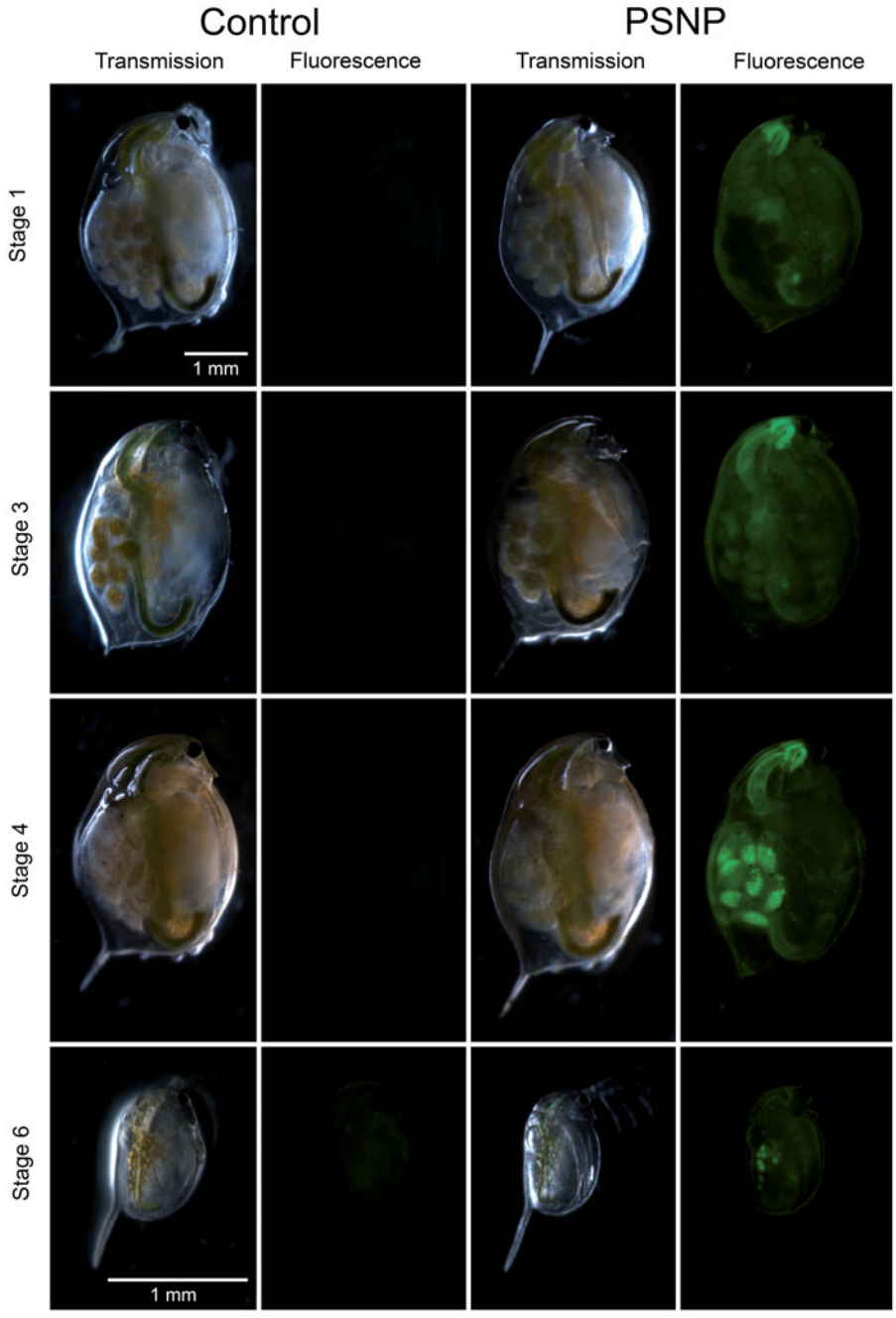

Figure 3. Representative fluorescence microscopy images of different stages of *Daphnia magna* development ($n = 8$). Adult daphnids were exposed to 5 mg L^{-1} PSNP from the moment that their brood pouch was empty until their embryos were in stage 4. GFP filter settings were an exposure duration of 2.5 s, a gain of 2.0 and a gamma of 0.6. Stage 1: the adult daphnid took up PSNPs but the embryo has not. Stage 3: the developing embryos took up PSNPs. Stage 4: PSNPs are concentrated in droplets in the embryo. Stage 6: neonate that has stored PSNPs in its fat droplets.

compounds have different mechanisms to pass membranes. While cellular uptake of lipophilic molecules is mainly receptor-mediated, lipophilic NPs are internalized through endocytotic uptake mechanisms. Brood pouches with active water circulation are found in many abundant and cosmopolitan members of aquatic amphipods and isopods (Hoese 1984; Dick, Faloon, and Elwood 1998; Seidl, Pirow, and Paul 2002). This suggests that the uptake mechanisms presented here are likely relevant for many aquatic organisms and should be accounted for in future studies. Moreover, these organisms present a significant link in aquatic food webs, highlighting the potential for trophic transfer.

Actual mechanisms underlying PSNP accumulation during embryogenesis remain uncertain. PSNPs

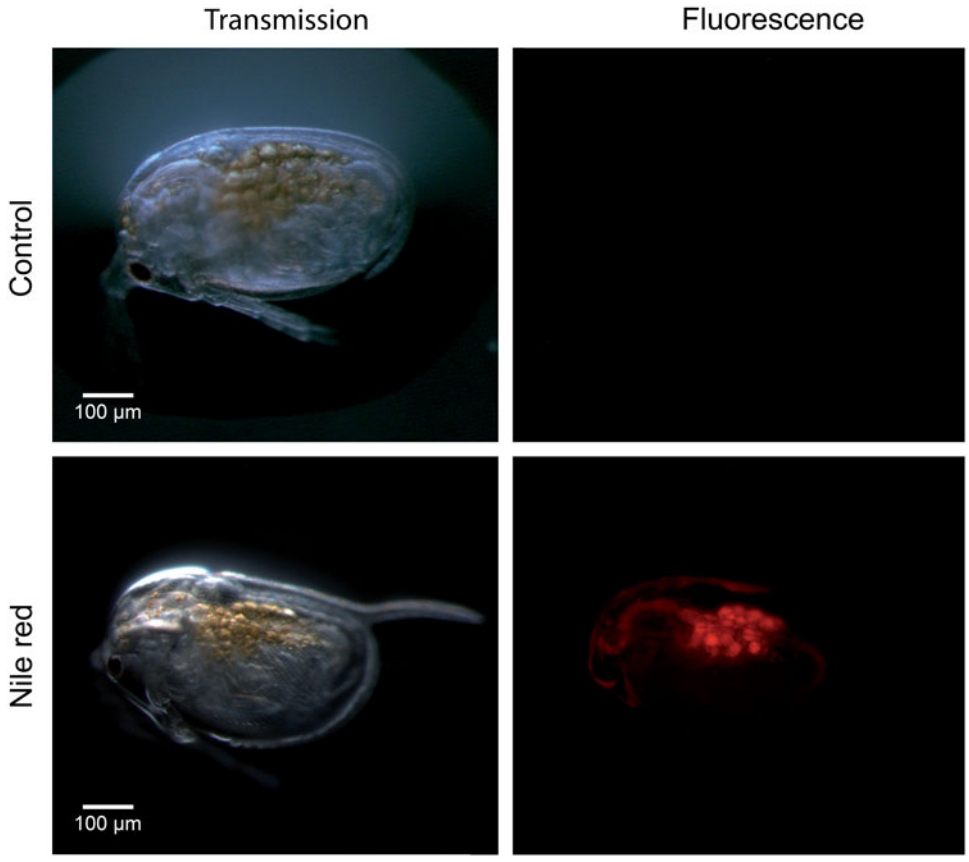

Figure 4. Representative fluorescence microscopy images of neonate *Daphnia magna*. The yellowish droplets in the central part of the body are fat droplets, as confirmed by staining animals with the lipophilic dye Nile red (bottom picture). DsRED filter exposure settings were an exposure duration of 448.9 ms, a gain of 2.0 and a gamma of 0.6.

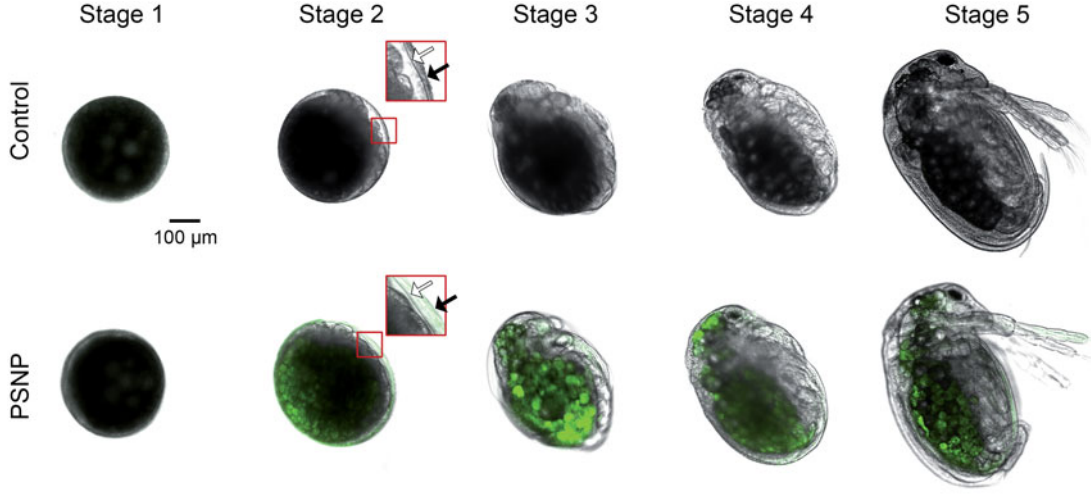

Figure 5. Representative confocal fluorescence microscopy images of *Daphnia magna* embryos during *ex vivo* exposure ($n = 24$). Overlay images of bright field and excitation at a wavelength of 488 nm are depicted of stage 1 to 5 showing uptake of PSNP (green fluorescent) from stage 2 onwards. Embryos at stage 2 have an inner (white arrow) and outer (black arrow) chorion, the latter bursts before stage 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

may adhere to the surface of the lipid droplets or be internalized. Both are described for polymer microparticles on lipid membranes (Van der Wel, Heinrich, and Kraft 2017). A more thorough understanding of the exact localization of PSNPs in or on lipid droplets, respectively, might be of interest for future studies. Here, we observed that the eggs start to internalize PSNP after being in the

brood pouch from stage 2 onwards, thus in early embryogenesis while the embryos are still in the chorion shell (Mittmann et al. 2014; Giardini, Yan, and Heyland 2015). This suggests that, in contrast to the observed protective role of chorion in fish (Fent et al. 2010), the chorion of crustacean embryos does not play a protective role for NP exposure. *Daphnia* embryos were observed to accumulate PSNP before organogenesis begins, suggesting that PSNPs or xenobiotics in general, may thus affect early embryonic development. While this is the first study showing accumulation of PSNP in early embryonic stages suggesting cellular accumulation or adhesion, Rosenkranz et al. (2009) also observed storage of fluorescent particles with a size of 20 and 1000 nm at a stage shortly before hatching, suggesting that the onset of filtering activity within the brood pouch presents a source for accumulation. Retention time of PSNPs in embryos was longer than retention time of PSNPs in guts of adults, indicating cellular internalization or adherence results in longer exposure to PSNPs than ingestion.

NP-contamination in complex environments typically results in a mixture of freely dispersed and adsorbed NPs. In this study, NPs were adsorbed to algal cells, as frequently observed in other studies (Hartmann et al. 2013; Nolte et al. 2017; Sørensen et al. 2016). It can be expected that NPs excessively covering algal cells are more likely ingested by filter feeding organisms since the mesh of the filtering apparatus is developed to concentrate particle in the μm-range such as algal cells. Nevertheless, also without the presence of food, NPs are found in the gut (Rosenkranz et al. 2009). NPs adsorbed to algae may result in either harmless gut-passage when the algae remain undigested (e.g. Porter 1975), or increased exposure concentrations in digestive tracts when the algae are digested. In contrast, freely dispersed NPs in aqueous environments are more likely taken up by embryos. It is thus possible that different fractions might be relevant for different uptake routes, and this potentially explains our observation that the ingestion of algae-adhered PSNP did not result in maternal transfer of large PSNP quantities. In addition, this also suggests that actual exposure concentrations of the dispersible PSNP fraction leading to accumulation in embryos are likely lower than nominal exposure concentrations.

Whether the observed brood pouch-mediated PSNP uptake ultimately translates to long-term effects under chronic exposure to environmentally relevant concentrations remains a challenging area for further research. In this study, concentrations used for preliminary 21-day chronic exposures did not result in mortality or reproductive impairment of *Daphnia* exposed from neonatal stage to adulthood, in which embryonic exposure was not captured. Whether the disintegration of the fat droplets and thus release of the PSNPs bears fitness consequences (e.g. growth) for the offspring or whether the PSNPs are excreted without further harm thus remains unknown in this study. *D. magna* exposed from the neonatal stage for 21 days to PSNPs (100 nm, 1 mg L^{-1}) responded with a decreased feeding rate while reproduction was not affected (Rist, Baun, and Hartmann 2017). In contrast, smaller PSNPs (70 nm) caused lower numbers of neonates and reduced body size (0.22 mg L^{-1}) and with malformations (30 mg L^{-1}) in *D. magna* (Besseling et al. 2014). Both clutch and neonate body size are related to a general stress response that is also found for other contaminants, indicating the ecological relevance of the compound exposure. Likewise, in sea urchin embryos, amino PSNP (50 nm; 3 mg L^{-1}) exposure disrupts development and induces teratogenic effects as well as stress genes and proteins (Della Torre et al. 2014; Pinsino et al. 2017). In addition, copepod nauplii can show clear ingestion of PSNPs, although dorsal imaging of the animals does not allow to distinguish between gut and oil sac serving as lipid storage. This ingestion of fluorescently labeled PSNPs (50 nm; 1.25 mg L^{-1}) was observed to cause ~90% mortality of nauplii in both the F_0 and F_1 generation (Lee et al. 2013).

Conclusion

Small plastic debris is ubiquitously found in the water column of aquatic ecosystems, and therefore planktonic filter feeders are particularly at risk. This potentially affects entire food webs considering the abundance of filter feeders and the wide array of organisms that prey upon them. While ingestion of PSNPs is currently considered the primary route for

cellular accumulation, here we illustrate a different route of NP accumulation, namely mediated through the brood pouch. This path of uptake has previously been overlooked. This new insight significantly furthers our understanding of PSNP accumulation. Moreover, this route of uptake likely occurs for other lipophilic compounds and in a wide array of aquatic organisms. Brood pouch-mediated contaminant accumulation from the surrounding water natural environments and exposure medium within experimental exposures should thus be considered in future ecological and ecotoxicological studies.

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Disclosure statement

The authors report no conflicts of interest.

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