

Christoph Schür ORCID iD: 0000-0001-5865-980X

Sinja Rist ORCID iD: 0000-0003-3002-0793

Martin Wagner ORCID iD: 0000-0002-4402-3234

C. Schür et al.

Dye leaches from florescent particles

## WHEN Fluorescence is not a particle: The tissue translocation of microplastics in *Daphnia magna* seems an artifact

Christoph Schür<sup>a,\*</sup>, Sinja Rist<sup>b,1</sup>, Anders Baun<sup>b</sup>, Philipp Mayer<sup>b</sup>, Nanna B. Hartmann<sup>b</sup>,  
Martin Wagner<sup>c</sup>

<sup>a</sup>Department of Aquatic Ecotoxicology, Faculty of Biological Sciences, Goethe University, Frankfurt am Main, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany

<sup>b</sup>Department of Environmental Engineering, Technical University of Denmark, Bygningstorvet, Building 115, 2800 Kgs. Lyngby, Denmark

<sup>c</sup>Department of Biology, Norwegian University of Science and Technology, Høgskoleringen 1, 7491 Trondheim, Norway

\*Address correspondence to schuer@bio.uni-frankfurt.de (CS)

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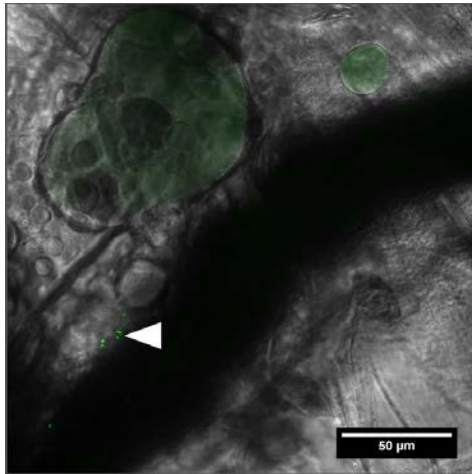
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<sup>1</sup> These authors contributed equally to this work

## Abstract

Previous research reported the translocation of nano- and microplastics from the gastrointestinal tract to tissues in *Daphnia magna*, most prominently of fluorescent polystyrene (PS) beads to lipid droplets. For particles  $> 300$  nm, such transfer is biologically implausible as the peritrophic membrane retains these in the daphnid gut. Thus, we aim at replicating the key study by Rosenkranz et al. (2009). We used confocal laser scanning microscopy to study the tissue transfer applying the original setup (neonates exposed to 20 and 1,000 nm PS beads at  $2 \mu\text{g L}^{-1}$  for 4 and 24 h), the same setup with a fructose-based clearing, and a setup with a 1,000-fold higher concentration ( $2 \text{ mg L}^{-1}$ ). We used passive sampling to investigate whether the beads leach the fluorescent dye. While the 1,000 nm beads were visible in the gut at both exposure concentrations, the 20 nm beads were detectable at  $2 \text{ mg L}^{-1}$ , only. At this concentration, we observed fluorescence in lipid droplets in daphnids exposed to both particle types. However, this did not co-localize with the 1,000 nm beads which remained visible in the gut. We further confirmed the leaching of the fluorescent dye using a passive sampler, a method that can also be applied in future studies. In summary, we cannot replicate the original study but demonstrate that the fluorescence in the lipid droplets of *D. magna* results from leaching of the dye. Thus, the use of fluorescence as a surrogate for particles can lead to artifacts in uptake and translocation studies. This highlights the need to confirm the stability of the fluorescence label or to localize particles using alternative methods.

## Graphical Abstract



## Keywords

nanoplastics, microbeads, microspheres, silicone, rubber, dye leaching

## Introduction

Synthetic polymer particles in the nano- and micrometer size range, now referred to as nano- and microplastics (Hartmann et al. 2019), have been used for decades to study various biological processes, such as the feeding preferences of zooplankton (e.g., Burns, 1968), since they are often regarded inert. Depending on their size, small particles can pass through biological barriers and enter tissues (Wright et al. 2013). The subsequent physical damage is one of the adverse effects engineered nanoparticles or nano- and microplastics may have on aquatic organisms (Rist and Hartmann 2018). Therefore, investigating this phenomenon and the underlying mechanisms is highly relevant.

Previous research mostly focused on tissue translocation of small particles in mammals (Jani et al. 1989; Jani et al. 1990; Jani et al. 1992; Walczak et al. 2015) with a human health or drug delivery focus. With growing concerns about plastic pollution, this focus

has shifted towards studying particle translocation in an ecotoxicological context. So far, more than 30 studies have investigated this in a range of species, most commonly in fish, crustaceans, and mollusks (Triebkorn et al. 2018). The majority of these studies reported a transfer of nano- and microplastics into tissues based on a visualization or measurement of fluorescence but without confirming that the fluorescent dye remained associated with the particles. It is well known from engineered nanoparticle research that dyes that are not covalently bound to the material can leach and cause artifacts (Kettiger et al. 2013; Rothen-Rutishauser et al. 2013). Within the analytical laboratory, reversible absorption of chemicals into and subsequent desorption from polymer beads and coatings is also well documented and massively exploited for Solid Phase Microextraction (SPME; Mayer et al., 2000), Solid Phase Extraction (SPE; Thurman and Mills 1998) and chromatographic separations on reverse phase high performance liquid chromatography (HPLC) columns (Davankov and Tsyurupa 2011). Here, it is well known that the release of absorbed chemicals can be triggered by the correct desorption medium and is affected by factors such as ionic strength and pH. The low pH in the daphnid intestinal tract (around pH 4.5; Smirnov (2017)), as compared to the medium, may thus be a relevant factor.

Rosenkranz et al. (2009) were the first to report the tissue transfer of nano- and microplastics in the freshwater cladoceran *Daphnia magna*. Due to a firm knowledge base and the wide use of this species as a standard test organism in ecotoxicology, it is now also widely used to study ingestion and toxicity of plastics (e.g., Ogonowski et al., 2016; Imhof et al., 2017; Rist et al., 2017; Scherer et al., 2017; Martins and Guilhermino, 2018). Rosenkranz et al. (2009) exposed *D. magna* for 30 min to 20 and 1,000 nm polystyrene (PS) beads ( $2 \mu\text{g L}^{-1}$ ) and observed fluorescence in the lipid droplets in both

adults and neonates. Transmission electron microscopy (TEM) seemed to confirm the presence of 1,000 nm beads in the lipid droplets but was inconclusive for the smaller particles. Based on this, the authors deduced that both particle sizes passed biological barriers and translocated from the digestive tract into the animals' tissue where they accumulated in lipid droplets. However, a biologically plausible mechanism for this observation is currently lacking.

A translocation of particles to the lipid droplets or other regions inside the daphnid body would have to follow a certain chain of events: ingestion of particles, passage across the peritrophic membrane, and transfer across the epithelium of the digestive tract, and transport to the target tissue. Importantly, the peritrophic membrane prevents a translocation of larger particles. Daphnids – like many other arthropods – produce this membrane in the foregut where it encloses the food pellets (with which it is excreted) to prevent mechanical injury and pathogen infiltration of the epithelium (Hansen and Peters 1998). The membrane consists of chitin microfibrils, polysaccharides and proteins (Georgi 1969) and is impermeable for particles  $\geq 327$  nm (Hansen and Peters 1998). Accordingly, a translocation of 1,000 nm microplastics in daphnids, as reported by Rosenkranz et al. (2009), seems biologically implausible and deserves re-assessment. Smaller particles, such as the 20 nm beads used in the same study, may pass the peritrophic membrane and can get in contact with the epithelium. A cellular uptake depends on particle size, concentration, surface modification, and charge and is plausible for 20 nm beads. The negative surface charge of the PS beads used by Rosenkranz et al. (2009) enhances the attachment to cell surfaces (Zhu et al. 2013). Particles can cross membranes passively (low nanometer range, Zhu et al. (2013)), via endocytosis (probably

limited to particles  $< 1 \mu\text{m}$ ) or persorption (particles  $< 150 \mu\text{m}$  in mammals; Volkheimer, 1974; Wright and Kelly, 2017). Following uptake by epithelial cells, an active transport mechanism would need to transfer the particles to the lipid droplets which are scattered throughout the body but mostly located ventral to the gut. As the lipid storage in cladocerans is poorly understood, the potential mechanism of a transfer of particles to lipid droplets remains unknown. However, the peritrophic membrane will be the major morphological barrier preventing particles larger than 300 nm from entering the body of cladocerans. Accordingly, reports on the tissue transfer of larger plastics are biologically implausible based on current knowledge.

Therefore, the aim of this study is to replicate the findings of Rosenkranz et al. using (1) their original study design, (2) a sample preparation approach with improved sensitivity, (3) 1,000-fold higher exposure concentrations, and (4) a passive sampling experiment to investigate a potential leaching of the fluorescent dye from the PS beads.

## Materials & Methods

### *Experimental design*

We conducted four experiments to replicate the study of Rosenkranz et al. (2009) on tissue translocation of nano- and microplastics in *D. magna* and to further investigate this phenomenon. Experiment I aimed at replicating the original experimental design by Rosenkranz et al. (2009) as closely as possible. Since they reported tissue translocation for both neonates and adults at all exposure durations (0.5, 1, 2, 4, 6, 12, 24 h), we decided to focus on exposing neonates for 4 and 24 h, only. The exposure concentration was  $2 \mu\text{g L}^{-1}$  for both particle types (20 and 1,000 nm) as in the original study. In

experiment II, we applied the same exposure conditions as in Experiment I but used a fructose-based clearing method (SeeDB) to enhance the detection of particles in the daphnids. In experiment III, the particle concentrations were increased to 2 mg L<sup>-1</sup> for each particle type, which is a 1,000-fold higher concentration than in the original study. Finally, Experiment IV was a passive sampling study with the PS beads used in Experiments I–III.

#### Plastic particles

The plastic particles were identical to the ones used by Rosenkranz et al. (2009), that is, 20 and 1,000 nm carboxylated PS beads (Fluospheres<sup>TM</sup>) purchased from ThermoFisher. The beads were labelled with fluorescein isothiocyanate (FITC,  $\lambda_{\text{Ex}}$ : 505 nm,  $\lambda_{\text{Em}}$ : 515 nm). They were provided in a suspension containing 2 % (w/w) solids which was stored at 4 °C in the dark. Immediately before the experiments, the suspension was sonicated for 30 min (UR1, Retsch GmbH) and subsequently dispersed in U.S. EPA reconstituted hard water (Smith et al. 1997) in concentrations of 2  $\mu\text{g L}^{-1}$  (Rosenkranz et al. 2009) or 2 mg L<sup>-1</sup>.

#### Daphnid maintenance

The experiments were conducted with the water flea *D. magna* (clone from Birkendammen, Denmark) which was cultured in U.S. EPA reconstituted hard water in glass beakers. Cultures were kept at 20 °C and a light/dark cycle of 12:12 h. Every beaker contained twelve individuals in 800 mL medium, fed daily with the green algae *Raphidocelis subcapitata* at a concentration of  $2.5 \times 10^5$  cells mL<sup>-1</sup>. The culture medium was renewed twice per week.

### *Exposure conditions*

Before the experiments, *D. magna* neonates (< 24 h old) were starved for 24 h to reduce autofluorescence of ingested algae. In each experiment (Table 1), daphnids were exposed to the 20 nm and 1,000 nm particles for 4 h and 24 h. While a concentration of 2  $\mu\text{g L}^{-1}$  was used in experiments I and II, the concentration was increased to 2  $\text{mg L}^{-1}$  in experiment III. Negative controls not containing plastic particles were included in each experiment. The exposure was conducted in triplicates in 100 mL glass beakers containing 80 mL medium and five neonates each. The beakers were covered with glass lids to avoid evaporation and kept at 20 °C in the dark to prevent bleaching of the fluorescent particles. A total of 120 daphnids was exposed to 2  $\mu\text{g L}^{-1}$  and 75 daphnids to 2  $\text{mg L}^{-1}$  (Table 1). Additionally, the experiments included 30 and 15 control animals. In experiment I, specimens were preserved in 10 % formalin according to the method by Rosenkranz et al. (2009). To increase visibility, we used an adapted version of the fructose-based clearing method SeeDB (see below) in experiments II and III.

### *Fixation and tissue clearing protocol*

We compared animals treated with 10 % formalin and an adapted version of SeeDB as a solvent-free clearing method (Ke et al. 2013). This procedure was chosen since previous experiments had shown that plastic particles dissolve when using solvent-based clearing methods (e.g., with benzyl alcohol/benzyl benzoate). Following the exposure, animals were rinsed twice by consecutive transfer to clean medium using a pipette. For the formalin fixation, five individuals per replicate were transferred to a glass vial containing 5 mL of a 10 % formalin solution and stored at 4 °C in the dark. For the SeeDB clearing, the specimens were preserved in glass vials with 5 mL of a 4 % para-formaldehyde (PFA) solution overnight. Subsequently, each individual was transferred into one well of a 96-well plate and transferred through a series of solutions with increasing fructose concentration (20, 40, 60, 80, and 100 % w/v). To reduce damage to the specimen due to repeated transfer the fructose solution was removed and replaced rather than transferring the specimen. For each step, 150  $\mu\text{L}$  fructose solution were added to each well and the



samples were kept at 4 °C in the dark for at least 4 h. Duration of each clearing step was reduced in comparison to the original protocol developed for whole mouse brains because of the smaller tissue size (Ke et al. 2013).

#### Confocal laser scanning microscopy

Samples were investigated using a Zeiss LSM780 (Carl Zeiss, Jena, Germany) confocal laser scanning microscope (CLSM) equipped with an argon laser (DPSS 561-10,  $\lambda_{\text{Ex}}$ : 488 nm, laser power: 0.025). We recorded two fluorescence channels: One to visualize the particles ( $\lambda_{\text{Em}}$ : 493–550 nm) and the other to visualize Nile Red stained (details see SI) lipid droplets in selected animals ( $\lambda_{\text{Em}}$ : 571–753 nm, details see SI) in addition to a brightfield image. The samples of each experiment were imaged using consistent settings with slight variations for the bright field digital gain, which did not affect the fluorescence signals (details see SI). In addition to single images taken at 10× (Plan-Apochromat 10x/0.3 M27, pinhole diameter: 89.89  $\mu\text{m}$ ), 20× (Plan-Apochromat 20x/0.8 M27), and 40× (EC Plan-Neofluar 40x/0.75 Ph2 M27) magnifications, we recorded focus stacks for each animal (details see SI). Images were processed in FIJI 1.52i (Schindelin et al. 2012).

#### Leaching experiment

In experiment IV, we investigated the leaching of the fluorescence dye from the PS beads and subsequent transfer to a synthetic acceptor phase. Medical grade silicone rubber sheets (127  $\mu\text{m}$  thickness, Technical Products Inc., Decatur, USA) were cut into strips of approximately 400 by 5,000  $\mu\text{m}$  using a box cutter. The strips were placed in particle suspensions mimicking the conditions of experiment III for 24 h (2 mg L<sup>-1</sup> of 1,000 nm beads in 80 mL U.S. EPA reconstituted hard water). Additionally, we also used a higher concentration of 200 mg L<sup>-1</sup> over 24 h in a miniaturized setting (200  $\mu\text{L}$  total volume,

diluted with ultrapure water). All strips were rinsed in ultrapure water after the incubation, placed on an object slide and imaged using settings consistent with the daphnid images ( $\lambda_{\text{Ex}}$ : 488 nm,  $\lambda_{\text{Em}}$ : 493–550 nm, 10× magnification).

## Results & Discussion

### *Replication of the Rosenkranz et al. study (experiment I)*

In daphnids exposed to 20 nm PS beads, it was not possible to distinguish between control and exposed animals after 4 and 24 h (Figure 1A and B). Accordingly and in contrast to the findings of Rosenkranz et al., we did not detect fluorescence in daphnids exposed to the 20 nm particles at  $2 \mu\text{g L}^{-1}$ . The 1,000 nm particles were clearly visible inside the digestive tract of most exposed animals (Figure 1 C, visible in 10 out of 15 animals after 4 h exposure, 12 out of 14 animals after 24 h). This is in line with previous studies, demonstrating that daphnids readily ingest nano- and microplastics (Jemec et al. 2016; Ogonowski et al. 2016; Rist et al. 2017; Scherer et al. 2017; Frydkjær et al. 2017; Canniff and Hoang 2018). We did not observe a difference regarding the amount of particles in animals exposed for 4 h compared to those exposed for 24 h. This is not surprising given the short gut retention time of microplastics in *D. magna* (Ogonowski et al. 2016; Scherer et al. 2017) that will result in a constant re-uptake of particles. A representative CLSM image of each specimen from experiment I is deposited on figshare (DOI: 10.6084/m9.figshare.7240469).

In their study, Rosenkranz et al. (2009) observed strong fluorescence of 1,000 nm beads in the digestive tract of all studied daphnids (further details in Rosenkranz, 2010). In contrast to our observations (Figure 1C), individual particles were not visible. This

implies that in their study either higher ingestion, a lower CLSM resolution or a digital amplification of the fluorescence signal during CLSM prevented the imaging of individual particles. More importantly, Rosenkranz et al. reported strong fluorescence in the lipid droplets of daphnids exposed to  $2 \mu\text{g L}^{-1}$  of both, 20 and 1,000 nm PS beads, and concluded that the particles had translocated and accumulated there. In contrast, we did not observe any fluorescence outside the digestive tract in the 60 specimens analyzed in experiment I. Thus, we were unable to reproduce the original findings of Rosenkranz et al. with identical experimental conditions and a large sample size.

The formalin-treated specimens were largely nontransparent (Figure 1A–C). At the same time, we observed a strong autofluorescence of the carapax in all animals from experiment I (Figure S1). Sheehy and Ettershank (1988) investigated autofluorescence in *Daphnia carinata* and found regions with a blue and a green excitation, the latter being in a comparable wavelength range as the one used for green fluorescence in our and other studies. This includes the gut lumen and embryos in the brood pouch with blue green fluorescence. Additionally, they reported a post mortem increase in fluorescence.

Accordingly, autofluorescence may be a confounding factor when studying the translocation of fluorescent particles that needs to be accounted for, for instance by imaging an adequate number of control animals. As Rosenkranz et al. (2009) did not provide images of such controls, it is impossible to evaluate whether autofluorescence interfered with their imaging analysis. Rosenkranz et al. partly account for that by using a quencher, however. Importantly, a number of CLSM settings can affect the (auto)fluorescence. While the authors kept the gain and offset stable, other settings (e.g., laser power, gain for each used channel, pinhole) remain unreported. Unless all images

are recorded with consistent settings, a fluorescence signal of a tentative plastic particle may be nothing but the result of, for example, an elevated digital gain. Here, the full and transparent reporting of the controls and the imaging settings is essential.

Rosenkranz et al. provided additional TEM images that show dark structures in lipid droplets in both size classes. They acknowledge the presence of granular structures in both control animals and those treated with 20 nm particles. Thus, a translocation of the smaller beads based on TEM remains inconclusive. For the 1,000 nm beads, Rosenkranz et al. observed dark, oval structures with a diameter of about 2  $\mu\text{m}$ . While the authors use that as major argument to support the idea of a tissue translocation, this is far from conclusive. For instance, in the TEM images provided in Rosenkranz (2010) the beads alone look somewhat distinct (spherical, 1,000 nm in diameter, fuzzy edges) from the structures observed in the lipid droplets. In any case, “visual” interpretation of TEM images may be prone to artifacts and misleading conclusions (Jensen et al. 2016) and the results by Rosenkranz et al. need to be followed up by in-depth TEM imaging.

### ***Tissue translocation with improved animal transparency (experiment II)***

In experiment II, 60 animals were exposed to PS beads under identical conditions as in experiment I. The specimens were cleared using the SeeDB method to increase their transmittance and, thus, the detection of fluorescent particles in the animals. The clearing improved the visibility (Figure 1D–F) compared to the original formalin fixation.

However, this treatment reduced sample integrity, increasing the risk of damaging the specimens during handling. In accordance with experiment I, no fluorescence was observed in daphnids exposed to 20 nm beads (Figure 1E). After 4 and 24 h exposure,

animals exposed to 1,000 nm beads had visible particles inside the gut (Figure 1F). No fluorescence in lipid droplets was observed in animals collected from either of the treatments. Therefore, an accumulation of nano- and microplastics in lipid droplets was not confirmed even when their detectability was improved.

***Tissue translocation with a 1,000-fold higher concentration (experiment III)***

Since no particle translocation was found in experiments I and II and the 20 nm beads were not detected at all under the experimental conditions used by Rosenkranz et al. (2009), we repeated the experiment with a 1,000-fold higher particle concentration (2 mg L<sup>-1</sup>). The SeeDB clearing was applied based on the improvement on particle detection described above. In contrast to experiments I and II, fluorescence was observed in the lipid droplets and guts of animals exposed to both, 20 nm and 1,000 nm beads and after 4 h and 24 h exposure (Figure 2). Accordingly, the observation of Rosenkranz et al. can be replicated using a 1,000-fold higher concentration. Interestingly, the fluorescence in the lipid droplets quickly faded during CLSM imaging, indicating a quenching or photo-bleaching of the dye. The latter is common for fluorescent dyes such as FITC (Johnson et al. 1982). As in experiment II, the 1,000 nm particles were clearly visible as individual beads in the gut of the daphnids (Figure S2). In contrast to the fluorescence in the lipid droplets, the fluorescence of the 1,000 nm beads in the digestive system was stable throughout imaging (Figure 3). To follow up, we investigated regions of interest covering the gut and lipid droplets at higher magnifications. Here, we could clearly differentiate between the fluorescent beads in the gut and the fluorescence in the lipid droplets which did not co-localize with any particles (Figure 4). Therefore, the fluorescence observed in the lipid droplets was not associated with the PS beads but probably caused by a leaching

of the fluorescent dye. As FITC is lipophilic (estimated  $\log K_{OW}$  of 4.69 according the U.S. EPA's EPI Suite (United States Environmental Protection Agency, Washington, DC, U.S.A. 2012)) it is probable that the chemical dye, and not the dyed particles, translocates from the gut and accumulates in the lipid droplets. This would also be consistent with the rapid loss of fluorescence during imaging. If true, the fluorescence observed in lipid droplets by Rosenkranz et al. (2009) is not proof of a tissue translocation of nano- and microplastics but merely an artifact caused by a leaching of FITC from the PS beads.

Notably, while plastic particles were not detected in lipid droplets, 1,000 nm beads were observed in close proximity but outside the digestive tracts of some daphnids (Figure S2). However, this was rare and always in the context of a damaged gut which was probably caused by the sample handling. Brun et al. (2017) made a similar observation in daphnids exposed to 25 nm PS beads and attributed particles outside the gut to damages during sample preparation.

#### Dye leaching experiment (experiment IV)

To test the hypothesis that the fluorescent dye is leaching from the particles, we incubated medical grade silicone rubber strips over 24 h with 2 and 200 mg L<sup>-1</sup> of 1,000 nm PS beads and subsequently imaged them using CLSM. After incubation with 2 mg L<sup>-1</sup> particles, the strips emit a weak fluorescence (Figure 5 A and A') compared to the control strip. Some particles could not be washed off and adhered to the surface but are clearly visible. A strip incubated with 200 mg L<sup>-1</sup> for the same period in ultrapure water exhibits a stronger fluorescence signal (Figure 5 C and C'), whereas the control strips did not (Figure 5 B and B'). These results indicate the transfer of FITC from the particles to a

synthetic matrix with similar partitioning properties as the lipid droplets in *Daphnia*. The 5 mm silicone rubber strips have a much higher volume than the lipid droplets in *Daphnia* neonates. Additionally, the dye transfer will continue after exposure of the animals until the subsequent imaging. In our case the strips were imaged immediately after exposure, but the daphnids were stored before imaging. Therefore, the weaker fluorescence signal in strips incubated with 2 mg L<sup>-1</sup> PS beads is likely due to a larger acceptor volume and a shorter incubation time. Accordingly, the fluorescence in lipid storage droplets in daphnids caused by a leaching of the dye from 2 mg L<sup>-1</sup> PS beads would be much stronger. This further supports that the fluorescence Rosenkranz et al. observed outside the digestive tract in fact might be an artifact caused by the leaching of the fluorescent dye. Interestingly, the shortcomings of using fluorescent dyes not covalently bound to particles have been discussed in the area of nanotoxicology (Kettiger et al. 2013; Rothen-Rutishauser et al. 2013). To avoid potential artifacts, future studies need to either demonstrate that the dye is not leaching under experimental or even digestive conditions (Gouliarmou et al. 2013). Alternatively, stably labeled particles e.g. with a metallic core (Mitrano et al. 2019) might be used or microscopic techniques that can certify the identity of the plastic particle by other means than fluorescence.

#### ***Are other studies affected?***

Following Rosenkranz et al.'s publication, a number of studies have shown micrographs indicating fluorescence in daphnia lipid droplets, even though investigating tissue translocation was not necessarily their primary objective. When exposing *Daphnia galeata* to 5 mg L<sup>-1</sup> of 51 nm green fluorescent PS beads, Cui et al. (2017) showed fluorescence inside embryos and lipid droplets, and concluded that there is a link to the

observed toxicity. Brun et al. (2017) investigated the brood pouch of *D. magna* as a potential exposure pathway for embryos, a mechanism proposed by Rosenkranz et al. (2009). They did not observe a translocation of 25 nm PS beads ( $5 \text{ mg L}^{-1}$ ) to maternal lipid droplets but into embryos. As the brood pouch is continuously flushed with water (Seidl et al. 2002), an exposure of embryos via this mechanism is probable. However, the mechanism for a transfer of particles into the embryo remains unclear. Here, the limitations in CLSM might lead to misinterpretation because particles adhering to the chorion cannot easily be distinguished from ones inside the embryo (e.g., in case of low resolution or strong fluorescence scattering). Chae et al. (2018) presented fluorescing lipid droplets in *D. magna* fed with algae that had been exposed to  $10 \text{ mg L}^{-1}$  of 51 nm fluorescent PS particles. The authors localized fluorescence inside a daphnid through z-stack projections, an approach that has a limited spatial resolution due to a blurring and scattering of the fluorescence signal across the z-axis. As none of the studies included controls for potential leaching of the fluorescent dye in vivo or during sample storage and imaging, it is not possible to evaluate whether it was indeed the fluorescent particles or just the fluorescent dye that translocated. Importantly, all studies used plastic particles with sizes  $<60 \text{ nm}$ , making translocation biologically plausible (see introduction). However, the imaging and, thus, the unequivocal localization of nanoplastics in tissues remains a fundamental methodological challenge.

Of the 31 studies Triebkorn et al. (2018) reviewed regarding tissue translocation of nano- and microplastics in aquatic invertebrates and fish over 75 % used polystyrene, mostly as commercially available particles. Additionally, fluorescence was the most widely utilized method to evaluate tissue translocation. Thus, the occurrence of false



positive results in other studies is plausible. However, as our experiments were performed with one type of particles only, it is not possible to generalize. The leaching and partitioning of dyes from other materials (e.g., different polymers) under other conditions (e.g., different media) remains to be investigated. Here, our passive sampling approach could be an effective screening method for the stability of dyes in commercial nano- and microplastics. Very recently, Catarino et al. (2019) published a study in zebrafish supporting the conclusion that dye leaching from 500 and 1,000 nm PS nanobeads causes artifacts. They suggested a dialysis step to remove uncoupled dye before performing toxicity studies. Dialysis was also recently presented as a method to account for biocidal additives to commercial particle suspensions (Pikuda et al. 2018). Taken together, these results highlight that fluorescent particles may not always be an appropriate surrogate for localizing nano- or microplastics in biological matrices.

## Conclusion

The potential translocation of nano- and microplastics into animal tissues is toxicologically relevant as it may cause internal mechanical injury, inflammation, and bioaccumulation. Previous research has postulated a transfer of 20 and 1,000 nm PS beads from the gut to the lipid droplets of *D. magna*. Since the biological mechanism for this phenomenon is implausible, at least for the larger particles, the aim of the present study was to replicate these previous findings. When using the original experimental setup as well as a method with improved sensitivity, we did not observe a tissue translocation of 20 and 1,000 nm PS beads at an exposure concentration of  $2 \mu\text{g L}^{-1}$ . When increasing the concentration by a factor of 1,000, fluorescence in lipid droplets was observed. However, this did not co-localize with the larger particles which remained in

the gut lumen. This implies that the fluorescence in the daphnid tissue was caused by the partitioning of the fluorescent dye from the plastic particles to the lipid droplets, which we confirmed using passive sampling. Accordingly, studies reporting a tissue translocation of nano- and microplastics using fluorescence imaging only, are prone to artifacts and need to be interpreted with caution in the light of biological plausibility. Strategies to minimize the risk for dye leaching artifacts in future particle uptake and translocation studies include: (1) Dye leaching during the experiment might be reduced by pre-washing the particles, (2) the absence of leaching at experimental or digestive conditions might be confirmed by simple passive sampling experiments and (3) the observed fluorescence within the tissue should only be taken as an initial observation that cannot stand alone to proof particle uptake and translocation.

### ***Supplemental Data***

The Supplemental Data are available on the Wiley Online Library at doi:

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### **Disclaimer**

The authors declare no conflict of interest.

### Author contributions

CS, NBH and MW conceived the study; CS, SR, AB, NBH, PM and MW designed the experiments; SR conducted the exposure and sample treatment of experiments I–III; CS conducted experiment IV, performed the CLSM imaging and analyzed the data; all authors interpreted the data; CS, SR and MW wrote the manuscript; all authors commented on the manuscript.

**Data accessibility**—Data pertaining to this manuscript are deposited in figshare at doi: 10.6084/m9.figshare.7240469. This article has earned an Open Data/Materials badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at 10.6084/m9.figshare.7240469. Learn more about the Open Practices badges from the Center for Open Science: <https://osf.io/tvyxz/wiki>.

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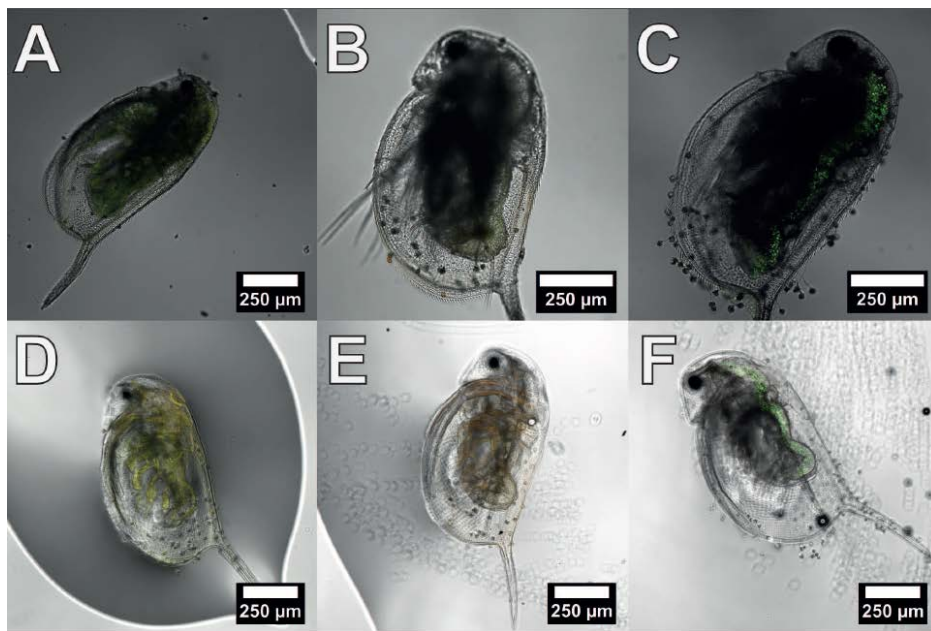
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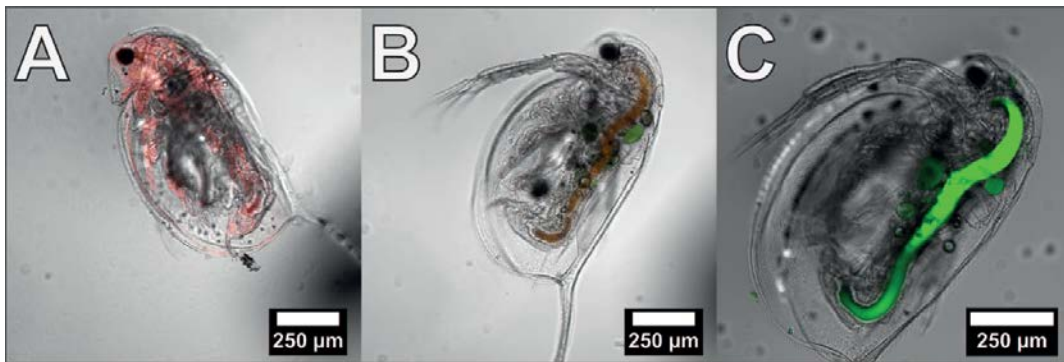
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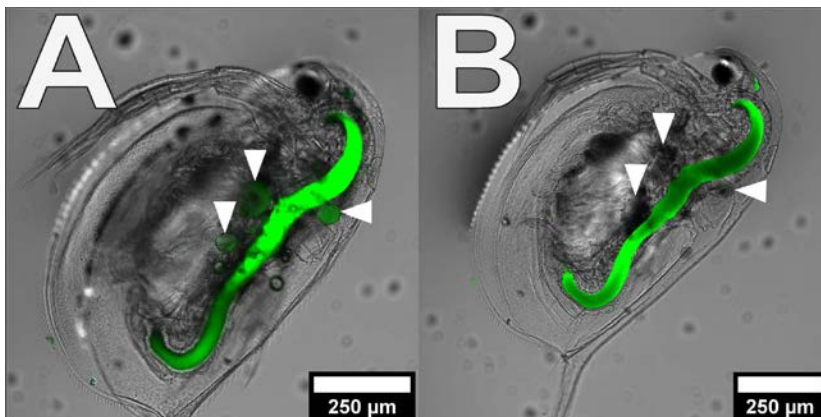
**Figure 1: Representative images of fixated *Daphnia magna* neonates exposed to  $2 \mu\text{g L}^{-1}$  fluorescent polystyrene particles using confocal laser scanning microscopy. A–C show specimens from experiment I that were treated identically to Rosenkranz et al.’s method (formalin fixation, experiment I). D–F represents daphnids treated with SeeDB clearing (experiment II). A+D: control animals, B+E: animals exposed for 24 h to 20 nm PS beads, C+F: animals exposed for 24 h to 1,000 nm PS beads. All images are single composite images extracted from a z-stack.**



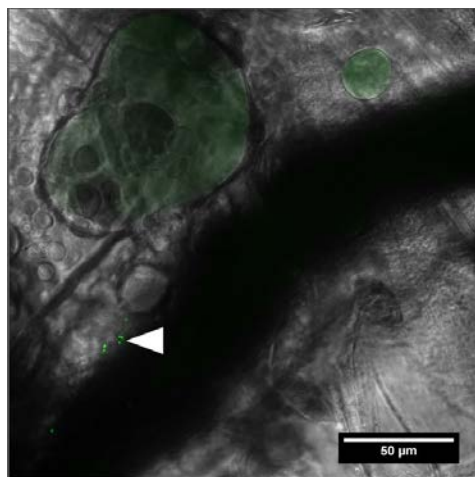
**Figure 2: Representative images of SeeDB-cleared *Daphnia magna* neonates exposed to 2 mg L<sup>-1</sup> fluorescent polystyrene particles using confocal laser scanning microscopy (experiment III). A: control animal, B: animal exposed for 24 h to 20 nm beads, C: animal exposed for 24 h to 1,000 nm beads. All images are single composite images extracted from a z-stack.**



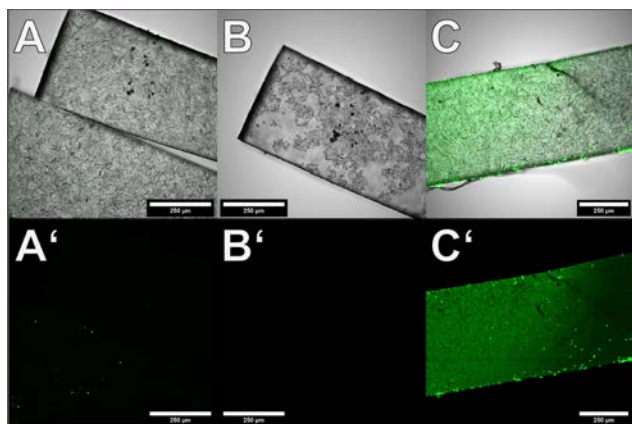
**Figure 3: Identical *Daphnia magna* individual exposed to 1,000 nm PS particles for 24 h before (A) and after (B) 60 min of confocal laser scanning microscopy imaging. The fluorescence is clearly visible in the lipid droplets (white arrows) initially (A) but faded during investigation (B). The particle-associated fluorescence in the gut lumen did not change. Microscope imaging settings are identical for both micrographs except for the zoom factor (0.8 in A, 0.7 in B) and a 26.2 μm difference in the z position.**



**Figure 4: Localization of 1,000 nm PS beads in the lumen of the daphnid gut (white arrow) and the fluorescence in the lipid droplet.** The fluorescence in the lipid droplets is not co-localized with the microplastics and quickly faded upon investigation.



**Figure 5: Transfer of fluorescent dye from 1,000 nm polystyrene bead suspensions (2 and 200 mg L<sup>-1</sup>) to silicone rubber strips in fluorescence and brightfield channel (A, B, C) and fluorescence only (A', B', C').** A+A': The upper and the lower strips were incubated for 24 h in U.S. EPA reconstituted hard water without and with plastic particles, respectively; B+B': Control strip incubated for 24 h in ultrapure water; C+C': Strip incubated for 24 h in 200 mg L<sup>-1</sup> bead suspension in ultrapure water.



**Table 1: Design of the three experiments to study the tissue translocation of polystyrene nano- and microplastics in *Daphnia magna*.**

<b>Experiment</b>	<b>Plastic particles</b>	<b>Concentration</b>	<b>Exposure duration</b>	<b>Clearing</b>	<b>Replicates (individuals)</b>
I (replication)	-	-	24 h	-	3 (15)
	20 nm	2 $\mu\text{g L}^{-1}$	4, 24 h	-	3 (15)
	1,000 nm	2 $\mu\text{g L}^{-1}$	4, 24 h	-	3 (15)
II (clearing)	-	-	24 h	SeeDB	3 (15)
	20 nm	2 $\mu\text{g L}^{-1}$	4, 24 h	SeeDB	3 (15)
	1,000 nm	2 $\mu\text{g L}^{-1}$	4, 24 h	SeeDB	3 (15)
III (higher concentration)	-	-	24 h	SeeDB	3 (15)
	20 nm	2 $\text{mg L}^{-1}$	4, 24 h	SeeDB	3 (15)
	1,000 nm	2 $\text{mg L}^{-1}$	4, 24 h	SeeDB	3 (15)