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Ingestion of micro- and nanoplastics in *Daphnia magna* – quantification of body burdens and assessment of feeding rates and reproduction

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Abstract

Evidence is increasing that micro- and nanoplastic particles can have adverse effects on aquatic organisms. Exposure studies have so far mainly been qualitative since quantitative measurements of particle ingestion are analytically challenging. The aim of this study was therefore to use a quantitative approach for determining ingestion and egestion of micro- and nanoplastics in *Daphnia magna* and to analyze the influence of particle size, exposure duration and the presence of food. One week old animals were exposed to 2 µm and 100 nm fluorescent polystyrene beads (1 mg/l) for 24 h, followed by a 24 h egestion period in clean medium. During both phases body burdens of particles were determined by measuring the fluorescence intensity in dissolved tissues. Ingestion and egestion were investigated in the absence and presence of food ($6.7 \cdot 10^5$ cells of *Raphidocelis subcapitata* per ml). Furthermore, feeding rates of daphnids in response to particle exposure were measured as well as effects on reproduction during a 21 days exposure (at 1 mg/l, 0.5 mg/l and 0.1 mg/l) to investigate potential impairments of physiology. Both particle sizes were readily ingested, but the ingested mass of particles was five times higher for the 2 µm particles than for the 100 nm particles. Complete egestion did not occur within 24 h but generally higher amounts of the 2 µm particles were egested. Animal body burdens of particles were strongly reduced in the presence of food. Daphnid feeding rates decreased by 21% in the presence of 100 nm particles, but no effect on reproduction was found despite high body burdens of particles at the end of 21 days exposure. The lower egestion and decreased feeding rates, caused by the 100 nm particles, could indicate that particles in the nanometer size range are potentially more hazardous to *D. magna* compared to larger particle sizes.

Capsule

The body burdens of plastic particles in *Daphnia magna* depended on the particle size, the exposure duration and the presence of food in the medium.

Keywords

microplastics; polystyrene; uptake; quantification; freshwater

1. Introduction

On a global scale aquatic ecosystems are polluted with plastic waste; ranging from lakes and rivers to coasts and the open ocean (Gregory, 2009). This includes microplastics (<5 mm), which stem from the fragmentation of bigger plastic items (i.e. secondary microplastics) or which have been produced in this size for different applications (i.e. primary microplastics) (Andrady, 2011). No lower size limit for particles formed by fragmentation has been described and therefore it is expected that also particles in the nanometer range (i.e. nanoplastics) are present in the environment. The term nanoplastics is not unambiguously defined and different studies set the upper size limit at 1 µm or 100 nm (da Costa et al., 2016; Koelmans et al., 2015). Either way it is due to analytical limitations at present not possible to detect, quantify and characterize these small plastic particles in environmental samples (da Costa et al., 2016).

The focus within microplastic research has so far mainly been on the marine environment, but an increasing number of studies show that microplastics are also widespread in freshwater systems (Wagner et al., 2014). As a result of their small size microplastics can be ingested by a large number of aquatic organisms. For marine species this has already been shown for many taxa (Besseling et al., 2015; Cole et al., 2013; Davison and Asch, 2011; Graham and Thompson, 2009; Watts et al., 2014; Wegner et al., 2012; Wright et al., 2013), whereas the number of studies on freshwater species is much smaller. So far ingestion of microplastic has been documented for the freshwater flea *Daphnia magna* (Besseling et al., 2014), the ostracod *Notodromas monacha*, the amphipod *Gammarus*

pulex, the oligochaete *Lumbriculus variegatus*, the mud snail *Potamopyrgus antipodarum* (Imhof et al., 2013) and different fish species (Khan et al., 2015; Oliveira et al., 2013; Rochman et al., 2013).

The ingestion of micro- and nanoplastics has been shown to induce oxidative stress and tissue damages in fish (Greven et al., 2016; Karami et al., 2016; Lu et al., 2016; Rochman et al., 2013), alter metabolism (Cedervall et al., 2012; Lu et al., 2016), lower the reproductive success (Besseling et al., 2014) and disturb normal behavior (Cedervall et al., 2012; de Sá et al., 2015). A set of studies with *D. magna* have shown that they readily ingest micro- and nanoplastics of various sizes (20 nm – 5 µm (up to 1400 µm in length)) and shapes, including beads, fragments and fibers (Jemec et al., 2016; Ogonowski et al., 2016; Rosenkranz et al., 2009). When *D. magna* were exposed to 70 nm polystyrene (PS) beads for 21 days Besseling et al. (2014) observed severe alterations of reproduction. For instance, an increase in neonate malformations occurred at concentrations above 32 mg/l. Microplastic exposure can also affect the feeding rates of *D. magna*. Total food intake decreased by 29% and 28% in the presence of 1-5 µm plastic beads and 2.6 µm polyethylene (PE) fragments, respectively, at a concentration of $2.25 \cdot 10^5$ particles/ml (Ogonowski et al., 2016). Furthermore, the particles modulated the growth of the daphnids. Immobilization of *D. magna* has been shown for a variety of micro- and nanoplastic particles, though the reported EC_{50} (concentration at which 50% of the daphnids were immobile) values range from 0.66 to 879 mg/l (Casado et al., 2013; Booth et al., 2015; Rehse et al., 2016). These values are difficult to compare since particle numbers are very different at the same particle mass concentrations for different particle sizes. Nonetheless, the observed toxicity in the listed studies varied considerably also for similar sized particles. This indicates that besides size the responses also depend on particle properties like polymer type and surface coating. Increased mortality was also observed during a 21 days exposure to $1 \cdot 10^5$ 2.6 µm PE fragments per ml. After 14 days 50% of the animals had died (Ogonowski et al., 2016). The reproductive success was, however, unaffected in this test.

Almost all ingestion studies, both on marine and freshwater species, have so far been qualitative. Quantitative analyses of plastic particle ingestion are analytically challenging

as carbon based particles are difficult to measure within organism tissues. In laboratory studies fluorescence is commonly used as a means of tracking and observing micro- and nanoplastics. With the use of fluorescence microscopy this allows the counting of particles within an organism, in dissected tissues or in the faeces (Hämer et al., 2014). Such a quantification technique is, however, limited by particle number and by sizes compared to the resolution and magnification limits of the microscope used. Another possibility as described by Rosenkranz et al. (2009) is to measure fluorescence intensity as a proxy for the particle amount.

For an assessment of potential effects of micro- and nanoplastics on organisms it is crucial to know to which extent they are able to ingest and subsequently egest the particles. This may depend on a variety of factors like the individual particle properties and the presence of other particulate and organic matter in the water. There is still only limited knowledge on how ingestion and egestion of micro- and nanoplastics depend on these factors. Furthermore, only few studies have analyzed effects on feeding and reproduction of daphnids and they did not link these responses to a quantification of ingested plastic particles. To explore this it is critical that appropriate sample preparation is applied, allowing for an accurate quantification of plastic particles inside the organisms. Therefore, the aim of this study was to quantify the ingestion and egestion of micro- and nanoplastic particles (2 µm and 100 nm, respectively) in *D. magna* in response to exposure duration and the presence of food in the medium, and to relate this to potential effects on feeding rates and reproduction. The hypotheses were: 1) *D. magna* ingests different sizes of particles in different quantities; 2) The ingested quantity of particles is lower in the presence of food; 3) The exposure to the particles leads to lower feeding rates; and 4) A 21 days exposure to the particles reduces the reproductive success of *D. magna*.

2. Materials and Methods

2.1 Experimental design

Within this study 4 main experiments were carried out. The experimental design of every experiment is presented here, while the methods are described in more detail in the following sections.

In experiment 1 the body burden of plastic particles, defined as the total particle mass per animal at a given time point, was measured during a 24 h exposure to the particles (ingestion phase), followed by a 24 h egestion phase in clean medium. Three factors were included in the test design: particles size (100 nm and 2 µm), sampling phase (ingestion and egestion phase) and exposure time until sampling (0.5, 1, 2, 4, 8 and 24 h). A diagram illustrating the setup is available in figure S1 (supplementary information). There were 3 replicates (n=3) per time point, resulting in 18 samples each for the ingestion and egestion phase, plus 3 controls of unexposed animals. The total number of samples was therefore 39 with each replicate consisting of 5 animals, resulting in a total of 195 animals per particle size (N=390 animals overall in experiment 1).

Experiment 2 investigated the influence of food presence on the body burden of particles. It had a similar setup as experiment 1, which is illustrated in figure S2 (supplementary information). The factors particle size (100 nm and 2 µm) and sampling phase (ingestion and egestion phase) were included as before. An additional factor was the presence of food with 2 levels (presence and absence). The exposure time was set to 24 h in both phases (without sampling in between). The 3 factors with 2 levels each resulted in 8 different treatment groups, of which each consisted of 3 replicates (n=3). There were again 5 animals per replicate, resulting in an overall number of 120 animals (N=120).

In the third experiment, which analyzed potential effects of particles exposure on *D. magna* feeding rates, only one factor ‘plastic exposure’ was implemented with 3 levels: no plastic particles, 100 nm particles and 2 µm particles. The feeding rate was determined by measuring the change of algal cell density within 24 h (see section 2.6).The number of replicates was 6, except for the 2 µm group, for which it was 5. Every replicate consisted of 5 one week old animals.

The fourth experiment was a 21 days exposure of *D. magna* to micro- and nanoplastics, looking at potential effects on growth and reproduction as well as analyzing the body burdens of particles at the end of exposure. The factors particle size (100 nm and 2 µm) and concentration (0.1, 0.5 and 1 mg/l) were included. Additionally there was one control

group, resulting in a total of 7 treatment groups. All animals came from the same population and were randomly assigned to the different groups. There were 10 replicates (n=10) consisting of 1 animal each per group. The overall number of animals was therefore 70 (N=70). During the 21 days different response variables related to growth and reproduction were measured and the body burden of particles was determined at the end of the test (see section 2.7).

2.2 Daphnia magna *cultures*

All laboratory tests were conducted with *Daphnia magna* (clone from Birkendammen, Denmark, 1978). Animals were cultured in glass beakers with Elendt M7 medium (OECD, 2008), which was exchanged twice per week, at a density of 12 animals per 800 ml. They were fed with the green algae *Raphidocelis subcapitata* daily ($2.5 \cdot 10^5$ cells/ml) and kept at a constant temperature of 20°C and a light-dark cycle of 12:12 h. For all tests, except the 21 days exposure test, one week old animals were used. For this purpose neonates (<24 h) were collected and cultured in separate beakers until the start of the test. Feeding took place as for the main cultures, except for the last 2 days before the test, in which the animals were not fed.

R. subcapitata was cultured in modified ISO 8692 medium (CEN, 2012) (enriched in nitrogen and phosphorus) under constant stirring, aeration and illumination until a cell density of approximately 10^6 cells/ml was reached and thereafter stored at 4°C in the dark. For feeding during the tests the algal cultures were concentrated to approximately 10^8 cells/ml by letting the cells settle at 4°C for 3-4 days and discarding the supernatant. This concentrated algae solution was used as a stock solution for feeding. Cell densities were determined using a particle counter (Coulter Counter Z2 and Multisizer 3, Beckmann Coulter) and the carbon content was calculated with the assumption that 10^7 cells correspond to 0.1 mg C (Halling-Sørensen et al., 1996).

2.3 Plastic particles

Fluorescent (Ex: 440 nm, Em: 486 nm) spherical PS beads with a size of 100 nm and 2 µm (density: 1.05 g/cm^3) were purchased from Phosphorex (www.phosphorex.com). They were supplied in deionized water with 0.1% Tween 20 and 20 mM sodium azide. The particle suspensions were stored at 4°C in the dark and vortexed before each use. The size distribution of the particles suspended in M7 medium was characterized using dynamic light scatter (DLS) (Zeta Sizer Nano Series, Malvern Instruments) for the 100 nm particles and particles counter (Multisizer 3, Beckmann Coulter) for the 2 µm particles. The mean size of the 100 nm particles was 95 nm (measured as hydrodanamic diameter, standard deviation (SD) =2.2 nm) and the polydispersity index (PDI) was 0.17 (SD=0.03), indicating a monodisperse suspension. Another measurement after 24 h in the suspension gave a slightly increased size of 168 nm (SD=12.08 nm) with a PDI of 0.26 (SD=0.02). The median size of the 2 µm particles was found to be 2.37 µm (SD=0.01 µm), which stayed unchanged during 24 h.

2.4 Quantification of animal body burdens of plastic particles

The fluorescence of the plastic particles was used to quantify the animal body burden of plastic particles, defined as the total particle mass per animal at the time of sampling. After sampling, the animals were rinsed with deionized water for approximately half a minute to remove all plastic particles from the surface. The complete removal was confirmed by fluorescence microscopy and by a pre-experiment in which dead daphnids were put into the particle suspensions. Then, after being sampled and rinsed in the same way as for the real test their tissues were digested and the fluorescence was measured. It was expected that any detectable particle fluorescence would stem from particles adhering to the animals' surface. However, no fluorescence was measurable, indicating that the rinsing procedure was sufficient to remove the particles from the surface.

In a pilot study it was found that a purely mechanical homogenization of the tissues resulted in a very low recovery of particle fluorescence. Therefore, different digestion protocols from the literature were compared for their interaction with the plastic particles

(100 nm PS) and efficiency to dissolve the daphnid tissues (all data is available in the supplementary information, see table S1). The different tested protocols used 65% nitric acid (HNO_3) (Vandermeersch et al., 2015), 1 M sodium hydroxide (NaOH) (Cole et al., 2014), 30% hydrogen peroxide (H_2O_2) (Mathalon and Hill, 2014), 25% tetramethylammonium hydroxide (TMAH) (Gray et al., 2013) and an enzymatic digestion using Proteinase K (Cole et al., 2014). The treatments with HNO_3, NaOH and H_2O_2 led to a loss of particle fluorescence as well as a strong agglomeration of particles and were therefore excluded. TMAH only lead to a slight decrease of particle fluorescence, but resulted in an incomplete dissolution of the tissues. The enzymatic digestion with Proteinase K gave the best results by completely retaining the particle fluorescence even though agglomeration was observed, and showing a highly efficient dissolution of the tissues (Table S1, Fig. S3). Therefore this protocol, adapted from Cole et al. (2014), was selected for all tests.

After sampling and rinsing, all animals of one replicate were transferred to one glass vial using a mesh and tissues were mechanically homogenized using a pestle tissue grinder (Tissue Grind Pestle 20, Kimble). Then 1 ml of a homogenizing solution, containing 400 mM Tris-HCl buffer, 60 mM EDTA, 105 mM NaCl and 1% SDS, was added and vials were incubated at 50°C for 15 min. This was followed by the addition of 0.23 µg Proteinase K and another incubation at 50°C for 2 h. After this, the vials were shaken at room temperature for 20 min and incubated at 60°C for 20 min. During all steps the glass vials were wrapped in aluminum foil to prevent a potential bleaching of the fluorescent particles. Immediately after the last incubation step the fluorescence in the medium was measured using a fluorescence spectrophotometer (F-7000, Hitachi). In order to deduct the particle quantity from the fluorescence measurement, a standard curve correlating particle concentration and fluorescence was made for each particle size. For this the fluorescence of a series of differently concentrated particle suspensions was measured after having gone through the whole enzymatic digestion protocol as described above. Good linear fits were found with R^2 values of 0.995 for both particle sizes and this was used to calculate particle concentrations from the measured fluorescence intensity (Fig. S1 and S2).

2.5 Ingestion/egestion tests – experiments 1 and 2

To investigate how the body burdens of particles change over time, *D. magna* (one week old) were exposed to plastic particles at a concentration of 1 mg/l for 24 h (ingestion phase), followed by a 24 h egestion phase in clean medium. During both phases *Daphnia* were sampled after 0.5, 1, 2, 4, 8 and 24 h, respectively. In terms of particle number 1 mg/l corresponded to $1.4 \cdot 10^5$/ml of the 2 µm and $3.1 \cdot 10^8$/ml of the 100 nm particles. During the test animals were kept in glass beakers in 15 ml M7 medium with the respective particle concentration. No food was added. The beakers were covered to avoid water evaporation and kept at 20°C in the dark. At the respective sampling time all 5 animals of one replicate were transferred to a mesh and rinsed with deionized water before the tissues were processed as described in 2.4. This was repeated for all replicates.

In the second experiment the influence of the presence of food on the particle body burdens was investigated. In the treatment groups with presence of food $6.7 \cdot 10^5$ cells of *R. subcapitata* per ml were added. The condition in which the beakers were kept as well as the sampling were identical to experiment 1.

2.6 Feeding rates of Daphnia magna *– experiment 3*

The feeding rates of *D. magna* were determined by measuring the depletion of algal cells in the test medium in 24 h. This was done with a particle counter (Multisizer 3, Beckmann Coulter). To check whether the cell density stayed constant without feeding there were control beakers without *D. magna* and only algae. All beakers were kept at 20°C in the dark during the whole test.

At the beginning of the test each beaker contained 18 ml M7 medium with an algal cell density of $1.5 \cdot 10^6$ cells/ml. This value was chosen to ensure that the cell density stayed above the incipient limiting concentration of $6.7 \cdot 10^5$ cells/ml during the whole test to keep the feeding rate constant (Furuhagen et al., 2014). The particle concentration was 1 mg/l. In the course of the 24 h test, water samples were taken to measure the cell density at 4 time points (t=0, 1, 19 and 24 h for 100 nm particles, t=0, 3, 8 and 24 h for 2 µm particles).

To obtain a homogeneous suspension for algal cell density measurements by particle counting, the animals were temporarily removed from the beaker using a pipette with a cut tip and transferred to a second beaker with clean medium. The test medium was then stirred on a magnetic stirrer for 20 sec before a sample of 900 µl was taken. The samples were immediately measured with the particle counter and the animals were put back into the test beaker.

In case of the 2 µm particles it was not possible to distinguish between PS particles and the algae cells in the particle counter due to the similarity in size. The counts therefore included both, leading to an overestimation of cell densities. However, in a pilot test measuring particle and algae suspensions alone and in combination, it was found that with the chosen concentrations the overestimation is only 5% (Fig. S8). Furthermore, we did not look at the single values but the rates, which are less affected by this error.

2.7 21 days exposure to micro- and nanoplastic particles – experiment 4

The 21 days exposure was conducted based on the OECD test guideline for the *D. magna* reproduction test (OECD, 2008). Individual daphnids were kept in 100 ml glass beakers with 50 ml M7 medium in a semi-static test setup, in which the medium was changed three times per week. The animals were fed with 10^7 cells of *R. subcapitata* per day and kept at 20°C in a light-dark cycle of 14:10 h. For both particle sizes concentrations of 1 mg/l, 0.5 mg/l and 0.1 mg/l were applied. When converted to particle numbers this corresponded to $3.1 \cdot 10^8$/ml, $1.6 \cdot 10^8$/ml and $3.1 \cdot 10^7$/ml for the 100 nm particles and $1.4 \cdot 10^5$/ml, $7 \cdot 10^4$/ml and $1.4 \cdot 10^4$/ml for the 2 µm particles, respectively. The exposure suspensions were freshly prepared before every medium exchange.

During the exposure several response variables relating to growth and reproduction were measured. These included the time to first offspring, the number of broods, the total number of neonates per daphnid, the number of neonates per brood, the number of molts, the mortality and the size of the mother animals at test end. The number of neonates and the mortality were checked daily. The number of molts was counted with every medium exchange.

At the end of the test the size of all surviving (adult) daphnids was measured using a stereomicroscope (M7_6, Leica Microsystems) with a connected camera (Go-5, Q-Imaging Inc.) and an imaging software (Q Capture Pro. 6.0, version 6.0.0.605 by Media Cybernetics Inc. and Q-Imaging Inc.). Following this, the animals were rinsed with deionized water and individually transferred to glass vials. The body burden of particles was determined for every daphnid as described in 2.4.

2.8 Data analysis

All graphs and statistical analyses were done with the free statistical computing software R (version 3.2.5 (2016-04-14)) (R Core Team, 2016). The effect of food presence in the ingestion/egestion test (experiment 2) was analyzed using a two-factorial ANOVA with the factors ‘sampling phase’ and ‘size’. The body burdens of particles at the end of the 21 days exposure (experiment 4) were analyzed with a two-factorial ANOVA with the factors ‘size’ and ‘concentration’. To check the assumptions of normality of errors and homogeneous variances, histograms and residual plots were used. Additionally the normality of residuals was tested with the Shapiro-Wilks-W-Test and the homogeneity of variances with the Fligner-Killeen Test. In case of the effects on food presence (experiment 2) and the body burdens after 21 days (experiment 4) the assumptions were violated, but this could be restored by transforming the data with the square root. The response variables of the 21 days exposure (except size of the mother animals) were analyzed using generalized linear models with a poisson distribution. In case of the number of neonates and the number of neonates per brood the ‘quasipoisson’ family object was used due to signs of overdispersion (the residuals deviance was much higher than the residual degrees of freedom). Significant findings between the groups were compared using the glht function in R (Bretz et al., 2010). The response ‘size of the mother animals’ was analyzed using a Kruskal- Wallis Test as a non-parametric alternative to ANOVA due to non-normality of the data. The feeding rates were analyzed with simple linear regression.

3. Results

3.1 Ingestion and egestion of micro- and nanoplastic particles – experiment 1

During a 24 h exposure to 100 nm and 2 µm particles, both sizes were readily ingested by *D. magna*. This was observed by microscopy as well as through analysis of the particle content in the exposed animals. The body burdens, defined as the particle mass per animal, increased with exposure time, reaching a steady state after approximately 4-8 h (Fig. 1). The maximum values were on average 0.17 µg/animal for animals exposed to 100 nm particles and 0.89 µg/animal for animals exposed to 2 µm particles. During the 24 h egestion phase body burdens stayed on a rather constant level; values of 0.12 µg/animal were found for the 100 nm particles and 0.57 µg/animal for the 2 µm particles. The patterns of the animal body burden in the course of the 48 h were similar for the two particle sizes, but the values differed greatly (Fig. 1). Expressed as particle mass per animal, the 'steady state' body burdens were approximately 5 times higher for the 2 µm particles, while the mass concentration in the exposure medium was the same. However, when calculated as particle numbers, the animals contained more than 400 times as many of the 100 nm particles in comparison to the 2 µm particles (Fig. S4 and S5, supplementary information). The difference in number concentrations in the exposure media for 100 nm and 2 µm particles, respectively, was by a factor of roughly 2200. Expressed as particles numbers maximum values during the ingestion phase were on average $5.29 \cdot 10^7$ particles/animal of the 100 nm particles and $1.24 \cdot 10^5$ particles/animal of the 2 µm particles. After the 24 h egestion phase these decreased to $3.88 \cdot 10^7$ particles/animal exposed to the 100 nm particles and $7.99 \cdot 10^4$ particles/animal exposed to the 2 µm particles (Fig. S4 and S5).

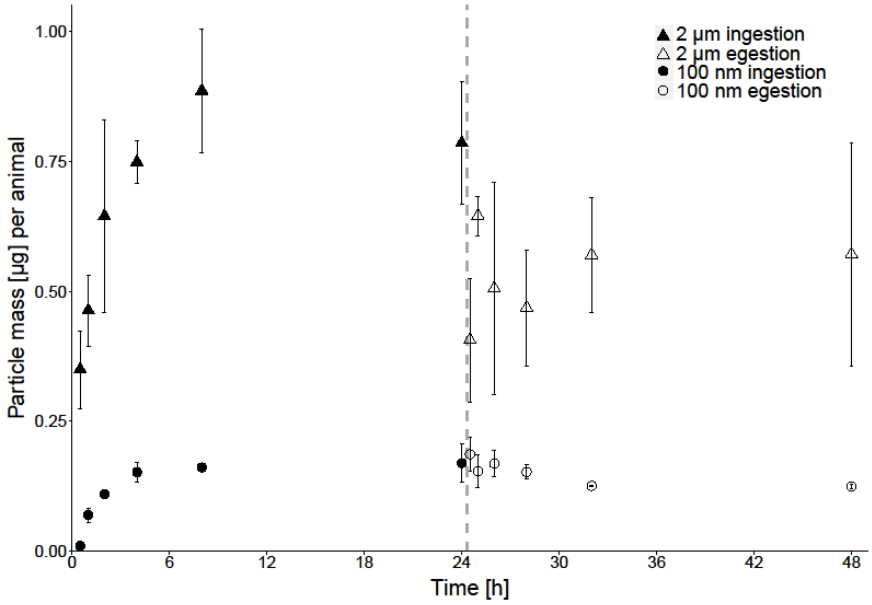

Fig. 1. Body burdens of particles (mass/animal) for *Daphnia magna* (1 week old) exposed to 100 nm (round symbols) and 2 µm (triangular symbols) polystyrene particles during a 24 h ingestion phase (closed symbols) in a particle suspension of 1 mg/l, followed by a 24 h egestion phase (open symbols) in clean medium. The mean and standard deviation for the 3 replicates per sampling point are shown.

3.2 Influence of the presence of food on animal body burdens of plastic particles – experiment 2

A test was done to investigate the influence of the presence of food on body burdens of particles after 24 h of ingestion and egestion, respectively. After the 24 h ingestion phase without the addition of food, average body burdens were 0.23 µg/animal for the 100 nm particles and 1.3 µg/animal for the 2 µm particles (Fig. 2). At the end of the 24 h egestion phase (without food) no significant decrease of the body burdens was found for the 100 nm particles. In contrast, the body burdens of animals exposed to the 2 µm particles decreased by 55%, reaching 0.59 µg/animal. When algal cells were added during the egestion phase, we observed a far larger decrease of body burdens. The 2 µm particles were no longer detectable in the animals after 24 h egestion. In the animals exposed to the 100 nm particles body burdens decreased by 93% during the egestion phase, reaching an average value of 0.02 µg/animal (corresponding to 9% of the body burden after egestion without food). When algal cells were added during the ingestion phase, we also observed significantly lower body burdens than in the absence of food. In the animals exposed to

100 nm particles the values were on average 78% lower (reaching 0.05 µg/animal) and in those exposed to the 2 µm particles the decrease was on average 98% (reaching 0.02 µg/animal). Both during ingestion and egestion the presence of food significantly decreased body burdens of particles and the two-factorial ANOVA found a significant influence of particle size, sampling phase and size:sampling phase interaction on daphnid body burdens of particles (Fig. 2, Table 1).

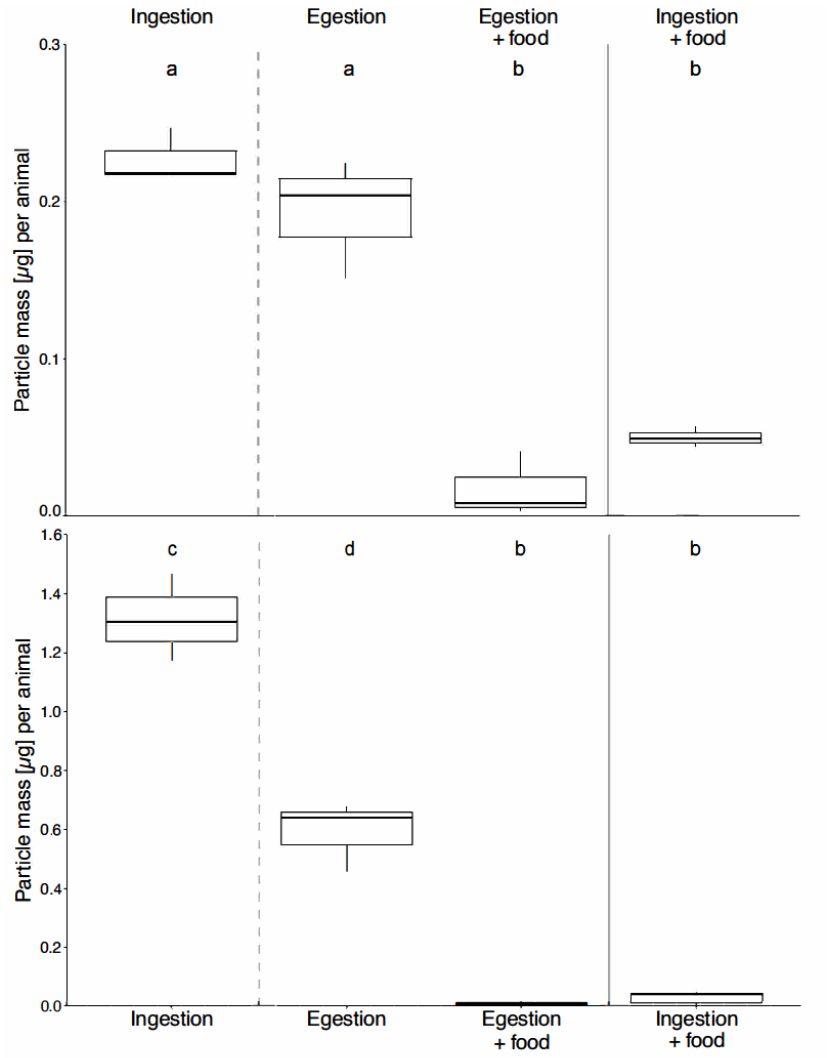

Fig. 2. Body burdens of particles (mass/animal) for *Daphnia magna* (1 week old) after 24 h ingestion (in particle suspensions of 1 mg/l) and 24 h egestion (in clean medium) of 100 nm (top) and 2 µm (bottom) polystyrene particles with or without the presence of algae in the medium. Groups that do not share the same letter are significantly different from each other.

Table 1

Results of the two-factorial ANOVA of the body burden of 100 nm and 2 µm particles (factor 'size') during a 24 h ingestion or egestion phase in the presence or absence of food (factor 'sampling phase'); including the degrees of freedom (d.f.), the mean squares (MS), the F-ratios and P-values.

	d.f.	MS	F-ratio	P-value
Size	1	0.563	197.15	$1.22 \cdot 10^{-9}$
Sampling phase	3	0.534	186.96	$1.58 \cdot 10^{-11}$
Size x Sampling phase	3	0.147	51.39	$8.39 \cdot 10^{-8}$
Residuals	14	0.003		

3.3 Influence of micro- and nanoplastic particles on feeding rates of Daphnia magna *– experiment 3*

The feeding rates of one week old *D. magna*, expressed as the number of ingested algal cells per time, are shown in table 2. In the group exposed to 100 nm particles the feeding rate was found to be significantly lower than in the control group (p=0.013) with a reduction of 21%.

Table 2

Feeding rates of *Daphnia magna* (1 week old) during 24 h exposure to algae only (control group) or algae in the presence of 1 mg/l of 100 nm or 2 µm polystyrene (PS) particles. Results of linear regression analysis are shown (SE=Standard Error).

Group	Feeding rate (ingested algal cells/h)	SE (cells/h)	R^2	P-value (diff. to control)
Control (algae)	$2.05 \cdot 10^4$	$2.0 \cdot 10^3$	0.97	-
Algae + 100 nm PS	$1.63 \cdot 10^4$	$1.6 \cdot 10^3$	0.82	0.013
Algae + 2 µm PS	$1.91 \cdot 10^4$	$1.9 \cdot 10^3$	0.85	0.093

3.4 Effects of 21 days exposure to micro- and nanoplastic particles – experiment 4

At the end of the 21 days test the animals of all treatment groups contained a measurable amount of particles. There was a significant effect of the particle concentration on the body burden and the pattern of body burdens followed the concentration in the exposure media with maximum values in the groups that were exposed to 1 mg particles/l (Fig. 3, Table 3). In the groups exposed to 100 nm particles we found on average 2.42 µg/animal for the highest exposure level (1 mg/l), 1.06 µg/animal for the medium level (0.5 mg/l) and 0.09 µg/animal for the lowest level (0.1 mg/l). In terms of particle numbers this corresponds to $7.6 \cdot 10^8$, $3.3 \cdot 10^8$ and $2.8 \cdot 10^7$ particles/animal. In the groups exposed to 2 µm particles the values were on average 2.43 µg/animal, 1.29 µg/animal and 0.1 µg/animal (corresponding to $3.4 \cdot 10^5$, $1.8 \cdot 10^5$ and $1.4 \cdot 10^4$ particles/animal), respectively. At the same particle concentration the body burdens were thus in terms of mass very similar for both particle sizes and we found no effect of particle size (Table 3).

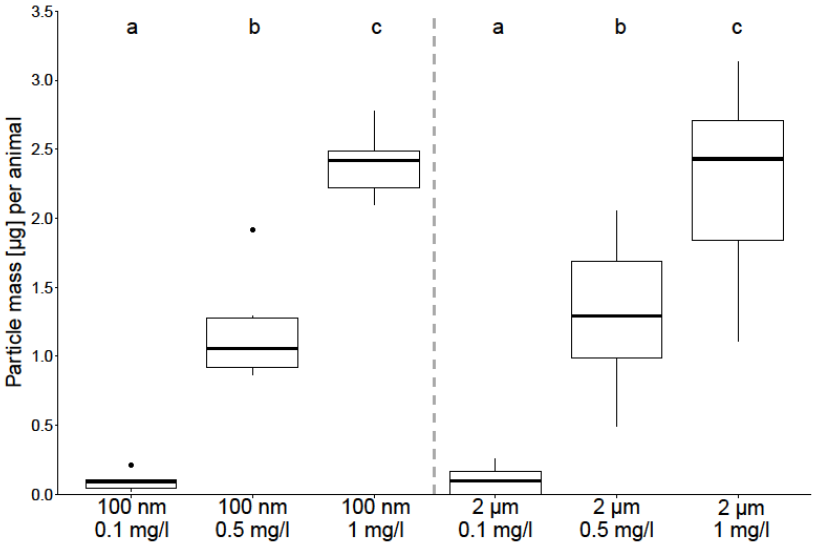

Fig. 3. Body burdens of particles (mass/animal) for *Daphnia magna* at the end of the 21 days reproduction test, in which animals were exposed to 100 nm or 2 µm polystyrene particles at different concentrations (0.1 mg/l. 0.5 mg/l, 1 mg/l). Groups that do not share the same letter are significantly different from each other.

Table 3

Results of the two-factorial ANOVA of the body burden after 21 days exposure to 100 nm and 2 µm particles including the degrees of freedom (d.f.), the mean squares (MS), the F-ratios and P-values.

	d.f.	MS	F-ratio	P-value
Size	1	0.016	0.52	0.475
Concentration	2	4.892	162.39	$2 \cdot 10^{-16}$
Size x Concentration	2	0.032	1.06	0.358
Residuals	38	0.030		

During the 21 days test no significant differences between treatment groups and the control were found for the following response variables: time to first offspring (Fig. S9), the number of broods (Fig. S10), the number of neonates per brood (Fig. S11), the number of molts (Fig. S12), the size of the animals at test end (Fig. S13) and the mortality (Table S2). Data for these test endpoints is available in the supplementary information. The total number of neonates produced within the 21 days of the experiment (Fig. 4) showed a trend of increasing numbers of neonates with higher particle concentration. This was especially pronounced for animals exposed to the 100 nm particles, e.g. a 56% increase (p=0.12) was found for animals exposed to 1 mg/l 100 nm particles compared to the control animals. However, there was no significant difference between the control and the other treatment groups.

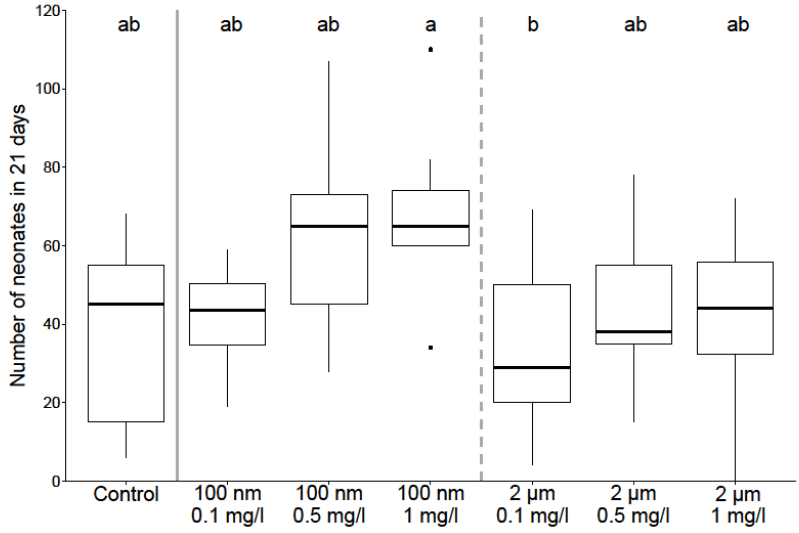

Fig. 4. Number of neonates produced per mother animal within the 21 days reproduction test, in which animals were exposed to 100 nm or 2 µm polystyrene particles at different concentrations (0.1 mg/l. 0.5 mg/l, 1 mg/l). Groups that do not share the same letter are significantly different from each other.

4. Discussion

In these tests animals were exposed to plastic particles in concentrations of up to 1 mg/l, which is much higher than measured environmental concentrations. However, the purpose of this study was to investigate the mechanisms and factors influencing particle ingestion under controlled conditions rather than testing environmentally relevant concentrations and settings. Both particles in the micro- (2 µm) and nanometer (100 nm) size range were readily ingested by *D. magna* under all tested conditions. Body burdens differed, however, based on particle size, exposure duration and the presence of food. *D. magna* constantly filter water to feed on small algae and organic matter, which explains the rapid increase of body burdens within the first few hours of exposure (Fig. 1). The 2 µm particles lie within the particle size range of 1-50 µm that *D. magna* usually feed on (Ebert, 2005)). The 100 nm particles are, however, smaller than the described minimum particle size of 0.24-0.64 µm for active filtration (Geller and Müller, 1981). Still, the ingestion of smaller particles down to 20 nm has been observed (Besseling et al., 2014; Casado et al., 2013; Cedervall et al., 2012; Rosenkranz et al., 2009; Skjolding et al., 2014). Gophen and Geller (1984) proposed that particles of nanometer size are caught by ultrafine structures of the filter combs or through interaction with the filter fibers. Furthermore, there may be passive uptake mechanisms. One possibility is that small particles interact with, and attach to, big particles like algal cells or detritus that are actively filtered or they could be ingested with water while drinking (Fox, 1952; Gillis et al., 2005). This potential difference of ingestion mechanisms for micro- and nanometer sized particles could explain the observed difference in body burdens. On a mass basis the body burdens were 5 times higher for the 2 µm particles compared to the 100 nm particles. Expressed as particle number, however, the amount of 100 nm particles was 400 times greater than for the larger particles, while the difference of particle number in the medium was roughly a factor of 2200. This implies that the ingestion of 2 µm particles was nonetheless more efficient, also measured on a number basis, with a larger fraction of the particles in the media being ingested. The uptake of 20 nm and 1 µm PS particles were compared by Rosenkranz et al. (2009), who also observed a much higher (by a factor 30 on a mass basis) ingestion of the bigger particles. However, the body burdens after 4 h of exposure were much lower than in this study: by a factor of almost 500 between the 1 µm particles in their study compared to our 2 µm particles, and a factor of 2500 between their 20 nm with our 100 nm particles. There

were essential differences between both studies, which could explain this. The exposure concentrations differed by a factor of 500 (2 µg/l vs. 1 mg/l) and the particle sizes, although in a similar range, were not the same, which limits the comparability especially in case of the smaller particles. Furthermore Rosenkranz et al. (2009) only conducted a mechanical homogenization of the tissues before measuring florescence, which we found to results in much lower fluorescence in comparison to the enzymatic digestion of tissues.

After a 24 h ingestion in experiment 1, no significant egestion of particles of both sizes occurred in the clean medium (without presence of food) and after 24 h considerable amounts of particles were still measured in the body. This may be attributed to the absence of food in the medium, since the pressure of new food in the gut system is needed for the egestion of faeces (Ebert, 2005). Consequently, in experiment 2 it was demonstrated that presence of food had a huge influence on body burdens, decreasing the particle mass per animal by 93% and 100% for the 100 nm and 2 µm particles, respectively, after 24 h in clean medium (with presence of food) (Fig. 2). The same pattern was also reported in a study with gold nanoparticles, in which *D. magna* was exposed to the particles during a 24 h uptake phase followed by a 24 h depuration phase, with or without food addition (Skjolding et al., 2014). A faster depuration in the presence of food was observed and the residual concentrations were dependent on particle size and higher for the bigger particles (30 nm in contrast to 10 nm). In our study, there was also a higher degree of egestion of the bigger particles, both in absence or presence of food, although the sizes and particle type differed greatly from the study by Skjolding et al. (2014). However, this difference has also been observed when comparing 20 nm and 1 µm PS particles, although the authors found a considerably higher egestion of both particles within 4 h in the absence of food (Rosenkranz et al., 2009) compared to this study. Particles in the nanometer size are more likely to get stuck in the gut system of *D. magna*, where surface structures (microvilli) are present in the foregut (Ebert, 2005). As a consequence they could persist in the organism for a longer time, which may increase their potential hazard.

When food was added during the ingestion phase the body burdens of particles were greatly reduced, by 78% and 98% for the 100 nm and 2 µm particles, respectively,

compared to ingestion without addition of food. Possible causes for this are that the plastic particles in the medium were ‘diluted’ with algal cells (the total particle number of algal cells plus plastic particles was almost by a factor 6 higher than plastic particles alone), naturally leading to a lower ingestion of the particles, and that the particle concentration in the medium decreased due to a more active filtration and thus deposition of particles in faeces. Another mechanism could be a certain degree of selective feeding. While some studies describe *D. magna* as a completely nonselective filter feeder (DeMott, 1986; Weltens et al., 2000) others observed some selectivity (DeMott, 1995; Gerritsen and Porter, 1982; Kirk, 1991). Kirk (1991) found selective feeding of phytoplankton over clay particles, which was described as passive selectivity since the animals capture them less efficiently instead of actively rejecting the inorganic particles. It has also been observed that *D. magna* is able to discriminate particles of different hardness and surface properties (DeMott, 1995; Gerritsen and Porter, 1982). Thus, there could have been differences in the ingestion rate and efficiency for algal cells and PS particles in this study. It is striking that the body burdens after the 24 h ingestion phase in the presence of food were closer to each other (0.05 µg/animal and 0.02 µg/animal for the 100 nm and 2 µm particles, respectively), in contrast to a factor 5 difference in the absence of food. This might again be attributed to several factors. The 100 nm particles could have interacted with the algal cells or formed aggregates in their presence, leading to a more active ingestion of this particle size. Furthermore, they could have accumulated in the gut system due to the lower egestion in comparison to the 2 µm particles, resulting in a gradual increase within 24 h.

During the 24 h feeding test (experiment 3), feeding rates were found to be constant (Table 2), which was expected at the chosen algae concentration that stayed above the incipient limiting concentration throughout the test (Ebert, 2005; Furuhagen et al., 2014). The measured control feeding rate of $2.05 \cdot 10^4$ cells/h, corresponding to 0.2 µg C/h, is lower than reported in other studies. Furuhagen et al. (2014) found a maximum rate of approximately 1.1 µg C/h, Taylor et al. (Taylor et al., 1998) reported a rate of $9 \cdot 10^4$ cells/h and Ogonowski et al. (2016) even described values of 37 µg C/h. However, the first study used adult daphnids, while the animals for this study were only one week old. Animal size, which was most likely different between the studies, strongly influences feeding rates, which can explain this discrepancy (McMahon, 1965). The animals of the studies by Taylor

et al. and Ogonowski et al. had a similar age, but also other factors like temperature and nutritional status can affect feeding rates (McMahon, 1965). However, the values by Ogonowski et al. (2016) lie far beyond our feeding rate as well as those reported in the other studies, which speaks for the influence of additional factors. Changes in feeding on natural prey, as a result of microplastic exposure, have already been described for a number of species, including the lugworm *Arenicola marina* (Wright et al., 2013), the Asian green mussel *Perna viridis* (Rist et al., 2016) and the marine copepod *Calanus helgolandicus* (Cole et al., 2015). So far one study looked at *D. magna* and reported feeding rates to decrease by 29% when the animals were exposed to 4.1 µm plastic beads at a concentration of $2.25 \cdot 10^5$ beads/ml (Ogonowski et al., 2016). For the 2 µm particles that were used in this study no statistically significant effect on feeding rates was observed, although the particle size and number ($1.4 \cdot 10^5$ particles/ml) was similar to the study by Ogonowski et al. (2016) ($2.25 \cdot 10^5$ particle/ml). However, in the treatment group exposed to the 100 nm particles feeding rates did decrease by 21% compared to the control ($p=0.013$). The 2 µm particles were expected to disturb the feeding process more compared to the 100 nm particles due to their size, which overlaps with that of the algae. However, this was not observed in this study. The distinct effect of the 100 nm particles could have been caused by an interaction with the filter setae and/or the gut wall, which is more likely for smaller particles, thereby disturbing the feeding process. It should be noted that the particle concentration clearly exceeds current levels found in the environment and the observed effects of nanoplastics is therefore not expected in freshwater ecosystems. However, the results demonstrate that even particles in the nanometer size range have the potential to interact with and impair the feeding of daphnids.

Decreased feeding activity in the long term is likely to result in further impairments of physiology and fitness of organisms as it affects the energy budget and thus the whole metabolism (Cole et al., 2015; Wright et al., 2013). Reproduction is an essential response as it can affect whole populations and for *D. magna* it has also been recognized as a sensitive endpoint for toxicity tests. We hypothesized that the 21 days exposure to micro- and nanoplastics would reduce the reproductive success of *D. magna*. This was, however, falsified in our test. For most response variables we found no differences between the plastic treatment groups and the control. In case of the number of neonates there was

even a slight increase with increasing concentration of the 100 nm particles, which would speak for a stimulation of reproduction (Fig. 4). However, the effect was very small. In contrast, in a reproduction test with 70 nm PS beads at concentrations of 0.22-150 mg/l, Besseling et al. (2014) found a reduction in the number and size of neonates, while an increasing fraction of neonates showed malformations at higher particle concentrations. This was, however, only observed at concentrations above 32 mg/l, which far exceed our highest treatment level. Another study, investigating effects of microplastics on reproduction of *D. magna,* used concentrations of 10^2-10^5 particles/ml, with the lowest tested concentrations approaching environmentally realistic values, and found no effects on reproductive success when animals were exposed to 4.1 µm plastic beads or 2.6 µm PE fragments (Ogonowski et al., 2016). This, together with our findings, indicates that the reproduction of *D. magna* is rather robust to micro- and nanoplastic stress at and above environmentally realistic particle concentrations. This was the case even though the animals had considerable amounts of particles in their bodies at the end of the 21 days exposure test (Fig. 3). The body burden values correlated with the concentration of the exposure suspension and were by far higher (up to 2.43 µg/animal) at the end of the 21 days exposure than what was found in the 24 h ingestion test (up to 0.57 µg/animal), despite the presence of food. This can on the one hand be attributed to the difference in animal age (and corresponding size), which can result in differences of feeding rates by up to one order of magnitude (McMahon, 1965). On the other hand a long term exposure might lead to a steady buildup of body burdens, especially in case of the smaller particles that are not egested so effectively. Again, in this case it is remarkable that body burdens at the end of the 21 days exposure were almost identical for both particle sizes, similar to the observation after 24 h exposure in the presence of food (Fig. 2), which suggests the same underlying mechanisms as described before. This implies that the presence of food does not only affect the overall body burden of ingested particles, but also modulates the differences between particle types. Important factors are most likely diverse interactions of different particle types with algal cells and other organic matter in the medium. Smaller particles may for instance agglomerate to a larger degree in the presence of algal and animal exudates, which could increase their availability to daphnids (Nasser and Lynch, 2016).

The observations of lower egestion and reduced feeding rates caused by the 100 nm particles, lead to the conclusion that the smaller particles are potentially more hazardous. This is most likely related to the huge difference in number when comparing the same particle mass, which, for example, results in a much higher surface-to-volume ratio. The smaller size also makes them more prone to getting stuck in small structures of organs and tissues. It is not possible to measure nanoplastics in environmental samples yet, but the continuous fragmentation of plastic debris in the environment is suggested to result in an ever increasing number of smaller particles, with much higher particle numbers in the nanometer size than what is currently found in the micrometer range (Andrady, 2011; Koelmans et al., 2015; Mattsson et al., 2015). This, and the findings of the current study, underlines that more focus should be directed towards the biological uptake and effects of the smaller size fraction of plastic particles in aquatic environments.

5. Conclusion

The results of this study show that measuring fluorescence intensity of plastic particles is feasible and provides valuable data for quantification of animal body burdens of particles in laboratory tests. By this means it was found that the ingestion and egestion of micro- and nanoplastics depend on the exposure time, the particle size and the presence of food: 2 µm particles were ingested and egested in higher amounts than 100 nm particles and body burdens generally decreased in the presence of food. These findings support our first and second hypothesis (ingestion depends on particle size and is affected by the presence of food). Biological effects following particle exposure also differed for the two particle sizes. While no effects on reproduction were observed for both sizes, falsifying the fourth hypothesis, the 100 nm particles lead to a 21% decrease of feeding rates, which was not found for the 2 µm particles. Thus, the third hypothesis was only falsified for the 2 µm particles. Taking all results together it can be concluded that the 100 nm particles are potentially more hazardous (having slower egestion rates and decreasing feeding rates) and more research should be directed towards the biological effects of nanoplastics.

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Supplementary information

1. Experimental design and setup

Figures S1 and S2 illustrate the experimental design and setup of the experiments, which investigated the animal body burdens of particles during a 24 h ingestion and subsequent 24 h egestion phase. During the ingestion phase animals were exposed to 100 nm or 2 µm particles at a concentration of 1 mg/l (corresponding to $3.1 \cdot 10^8$ and $1.4 \cdot 10^5$ particles/ml, respectively). Thereafter they were transferred to clean medium for the egestion phase. During the experiments 5 one week old animals were kept in a beaker with 15 ml M7 medium (n=3). In experiment 1 the animals were sampled after 0.5, 1, 2, 4, 8 and 24 h in each phase to analyze how the body burden of particles changed over time (Fig. S1). No food was added. In experiment 2 the influence of the presence of food on the body burdens was investigated. For this animals were either only exposed to a particle suspension during the ingestion and clean medium during the egestion phase as before or with the addition of $6.7 \cdot 10^5$ cells of *Raphidocelis subcapitata* per ml (Fig. S2). Animals were then sampled after 24 h, respectively.

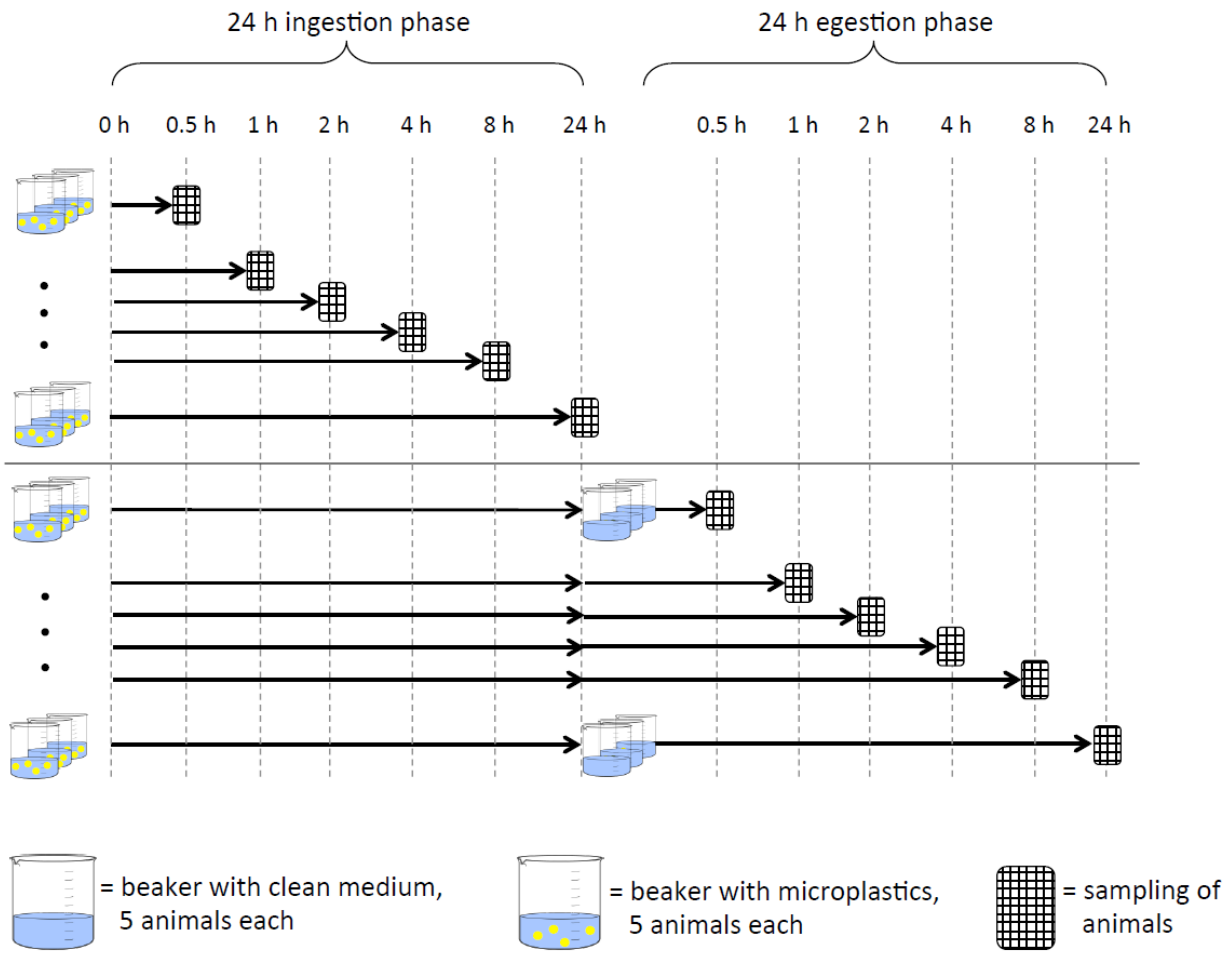

Fig. S1. Experimental design and setup of experiment 1. Daphnids were exposed to 1 mg/l of 100 nm or 2 µm particles (the figure only shows the setup for 1 particle size) during a 24 h ingestion phase, followed by a 24 h egestion phase in clean medium. During both phases animals were sampled after 0.5, 1, 2, 4, 8 and 24 h (2, 4 and 8 h are not shown but indicated by dots in the figure). Additionally, a control group was sampled directly at the start of the experiment (i.e. 0 h). There were 5 one week old animals per beaker and 3 beakers per treatment group.

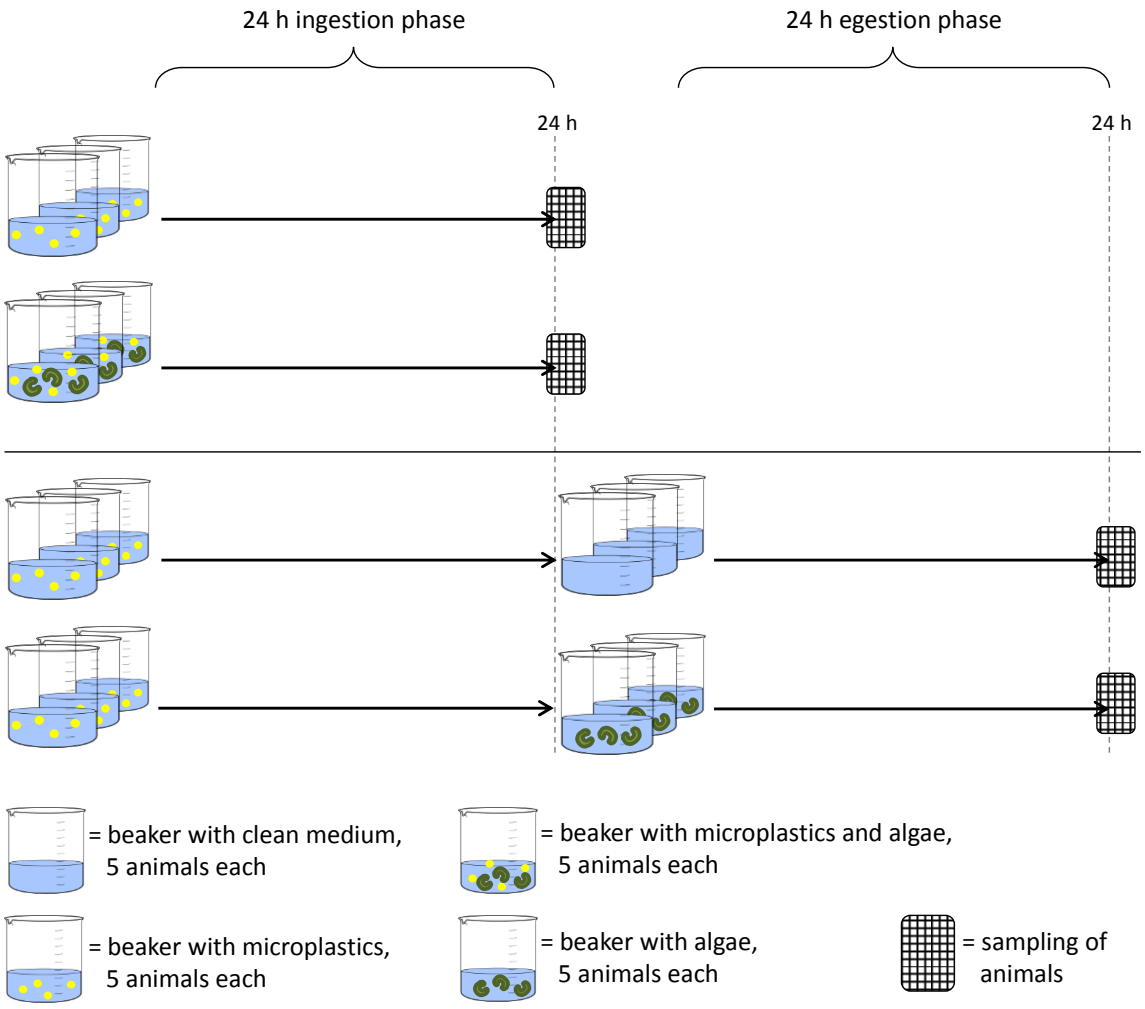

Fig. S2. Experimental design and setup of experiment 2. Daphnids were exposed to 1 mg/l of 100 nm or 2 µm particles (the figure only shows the setup for 1 particle size) with or without the addition of algae ($6.7x10^5$ cells/ml) during a 24 h ingestion phase, followed by a 24 h egestion phase in clean medium, again with or without algae. Animals were sampled after 24 h, respectively. There were 5 one week old animals per beaker and 3 beakers per treatment group.

2. Comparison of tissue dissolution protocols

To quantify the amount of polystyrene (PS) particles that was present in the organism at a certain time point, the fluorescence intensity in the whole tissue was measured. For this, animals were sampled, rinsed and mechanically homogenized using a pestle tissue grinder. However, only very low fluorescence signals were measurable in the tissue homogenate, since the cell and tissue debris most likely shaded a large fraction of the particles. Therefore different protocols to dissolve the tissues were tested: 65% nitric acid (HNO_3) (Vandermeersch et al., 2015), 1 M sodium hydroxide (NaOH) (Cole et al., 2014), 30% hydrogen peroxide (H_2O_2) (Mathalon and Hill, 2014), 25% tetramethylammonium hydroxide (TMAH) (Gray et al., 2013) and an enzymatic digestion using Proteinase K (Cole et al., 2014). They were compared for their efficiency to dissolve the tissues, which was checked visually (Fig. S3), and for their interactions with the particles. The latter focused especially on possible effects on the fluorescence intensity, but also on the agglomeration of the particles, which was analyzed by dynamic light scattering. The tests were done with the 100 nm particles (Table S1).

Table S1

Comparison of 5 different tissue dissolution protocols for their effects on particle fluorescence intensity and particle agglomeration, determined by the Z-average (nm) and the polydispersity index (PDI). Treatments with water served as controls. n=3, RT=room temperature

Solution	Protocol	Particle fluorescence	Z-Average (nm)	PDI
H_2O	24 h RT	0.402	98	0.15
H_2O	24 h 60°C	0.298	94	0.15
HNO_3 (65%)	24 h RT 1 h 60°C	0.056	846	0.70
NaOH (1M)	24 h 60°C	0.092	19972	0.76
H_2O_2 (30%)	24 h 60°C	0.008	3560	0.96
TMAH (25%)	24 h RT	0.297	246	0.13
Proteinase K	2.25 h 50°C 20 min RT 20 min 60°C	0.343	1426	0.30

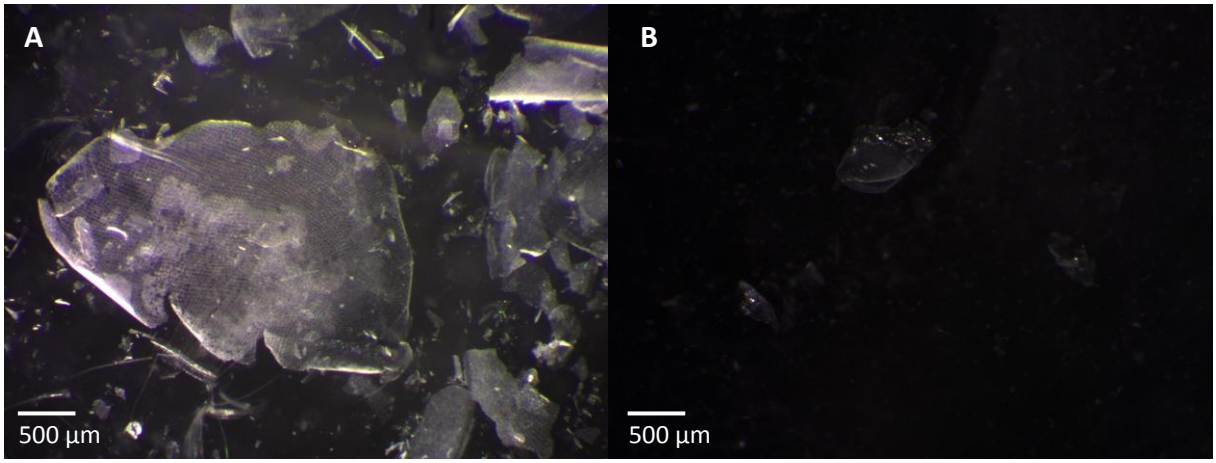

Fig. S3. Sample of *Daphnia magna* after only mechanical homogenization (A) and after mechanical homogenization plus enzymatic digestion (B). Pictures were taken with a stereomicroscope (M7_6, Leica Microsystems) with a connected camera (Go-5, Q-Imaging Inc.).

3. Standard curves for the measurement of animal body burdens

We measured the fluorescence of the PS particles to quantify the amount of particles that was present in the dissolved tissues of the animals. In order to calculate particles mass from the fluorescence intensity, a standard curve correlating particle concentration and fluorescence was made for each particle size (Fig. S4 and S5).

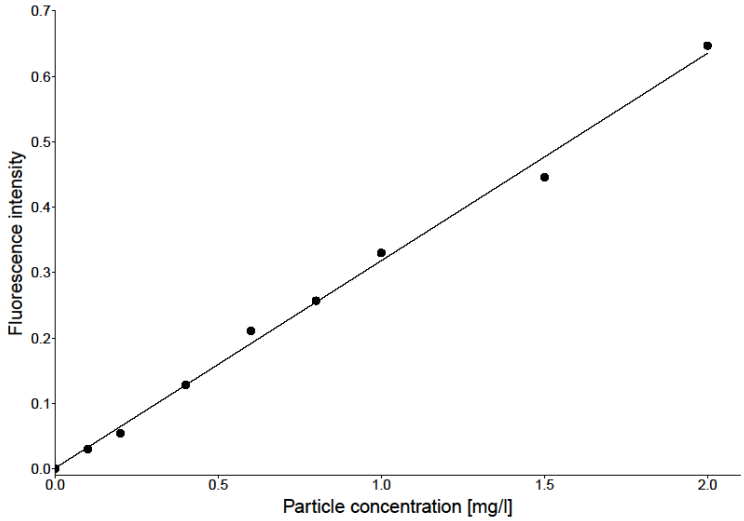

Fig. S4. Fluorescence intensity of the 100 nm polystyrene particles at different particle concentrations. The particle solutions were treated with the same enzymatic digestion protocol like the animal tissues before being measured. Linear regression analysis gave an R^2 value of 0.995. y=0.318x

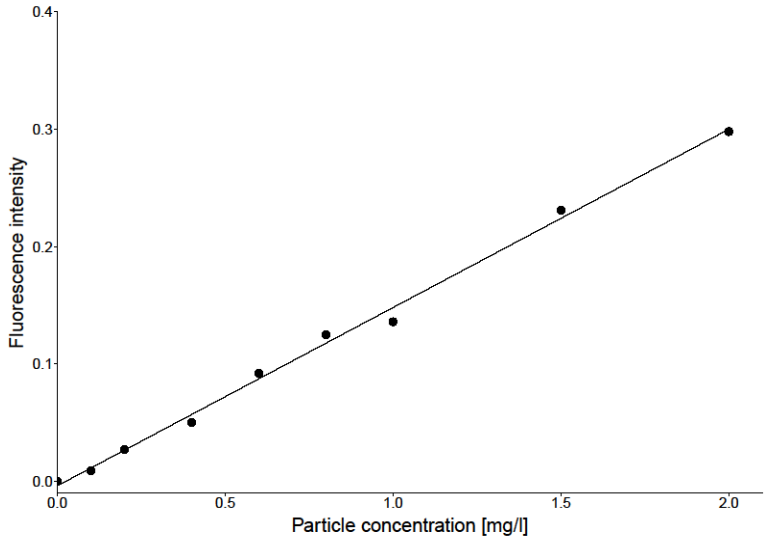

Fig. S5. Fluorescence intensity of the 2 µm polystyrene particles at different particle concentrations. The particle solutions were treated with the same enzymatic digestion protocol like the animal tissues before being measured. Linear regression analysis gave an R^2 value of 0.995. y=0.1489x

4. Ingestion and egestion of micro- and nanoplastic particles

The exposure levels in the tests were defined on a mass basis (mg/l) and subsequently also the body burdens of particles were calculated as mass per animal. However, we additionally calculated the body burdens as particle numbers per animal (Fig. S6 and S7). When comparing the two figures the different scaling of the y-axis should be noted.

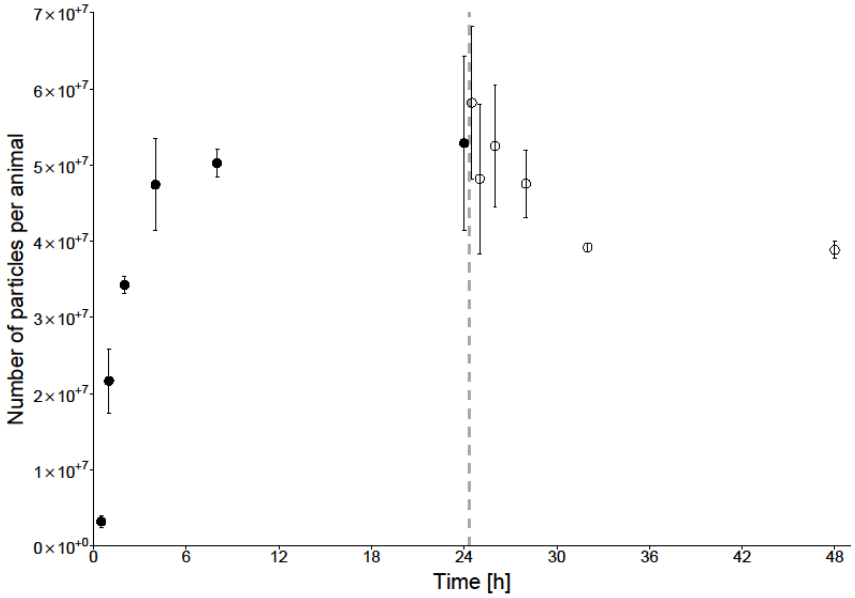

Fig. S6. Body burdens of particles (number/animal) for *Daphnia magna* exposed to 100 nm polystyrene particles during a 24 h ingestion phase (closed circles) in a particle suspension of $3.1 \cdot 10^8$ particles/ml, followed by a 24 h egestion phase (open circles) in clean medium. The mean and standard deviation for the 3 replicates per sampling point are shown.

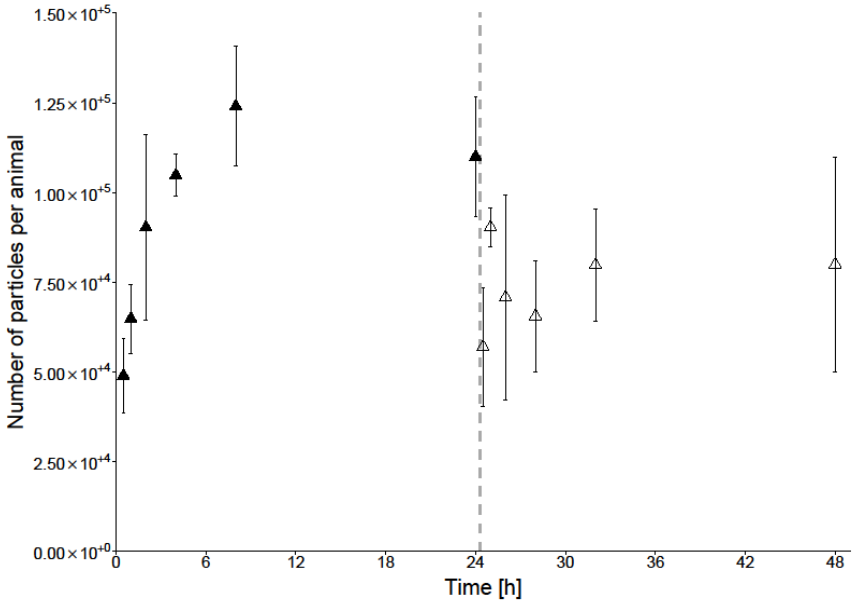

Fig. S7. Body burdens of particles (number/animal) for *Daphnia magna* exposed to 2 µm polystyrene particles during a 24 h ingestion phase (closed triangles) in a particle suspension of $1.4 \cdot 10^5$ particles/ml, followed by a 24 h egestion phase (open triangles) in clean medium. The mean and standard deviation for the 3 replicates per sampling point are shown.

5. Measurement of algal cells and 2 µm particles with the cell counter

For determining feeding rates of *D. magna* the depletion of algal cells in the medium was measured within 24 h. The cell density was analyzed with a cell counter (Multisizer 3, Beckman Coulter), which could, however, not distinguish between the 2 µm plastic particles and algal cells. Therefore, the counts included both, leading to an overestimation of the cell densities. To investigate the extent of this overestimation suspensions of the plastic particles and algae with the same concentrations as in the experiment were measured individually and in combination (Fig. S8). It was found that with the chosen concentrations the overestimation is only 5%.

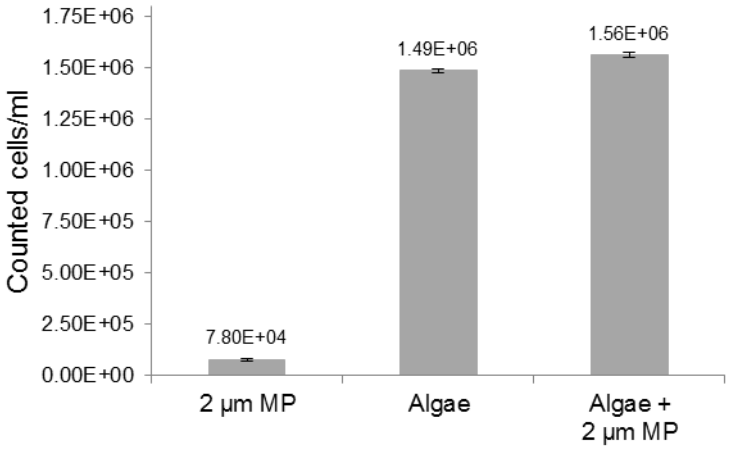

Fig. S8. Number of counted cells per ml in suspensions of 2 µm plastic particles (1 mg/l) and algal cells ($1.5 \cdot 10^6$cells/ml) alone and in combination (n=3).

6. Results of the 21 days exposure to micro- and nano-sized plastic particles

Different response variables relating to growth and reproduction were measured during a 21 days exposure of *D. magna* to both particle sizes at different concentrations. Here we show the data for: the time to first offspring (Fig. S9), the number of broods (Fig. S10), the number of neonates per brood (Fig. S11), the number of molts (Fig. S12), the size of the animals at test end (Fig. S13) and the mortality (Table S2).

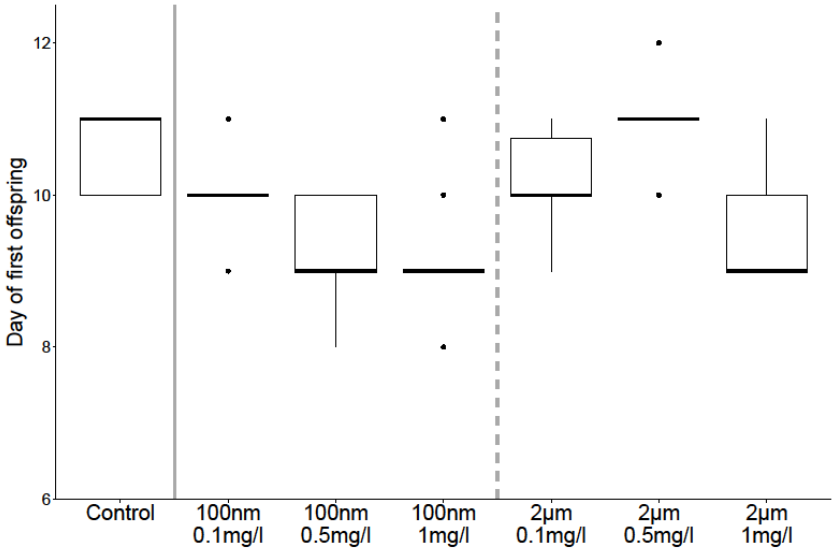

Fig. S9. Time to first offspring (in days) of mother animals within the 21 days reproduction test, in which animals were exposed to 100 nm or 2 µm polystyrene particles at different concentrations (0.1 mg/l. 0.5 mg/l, 1 mg/l). Groups that do not share the same letter are significantly different from each other.

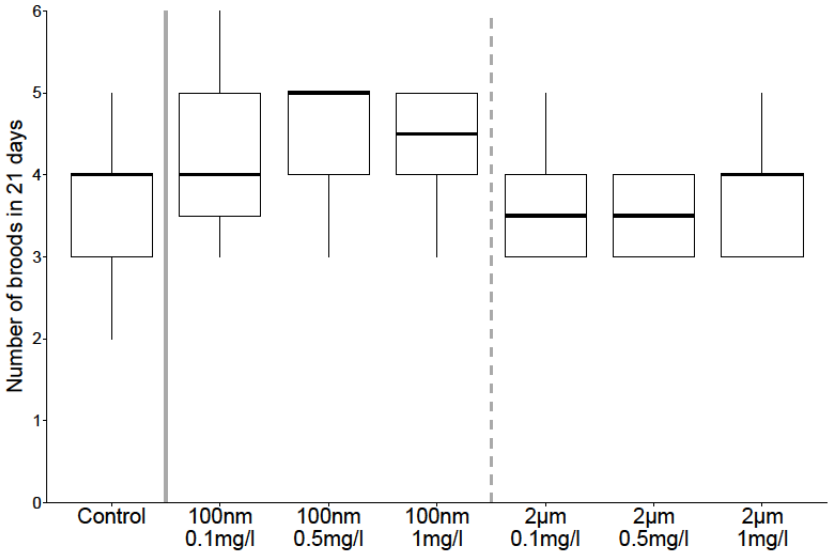

Fig. S10. Number of broods per mother animal within the 21 days reproduction test, in which animals were exposed to 100 nm or 2 µm polystyrene particles at different concentrations (0.1 mg/l. 0.5 mg/l, 1 mg/l).

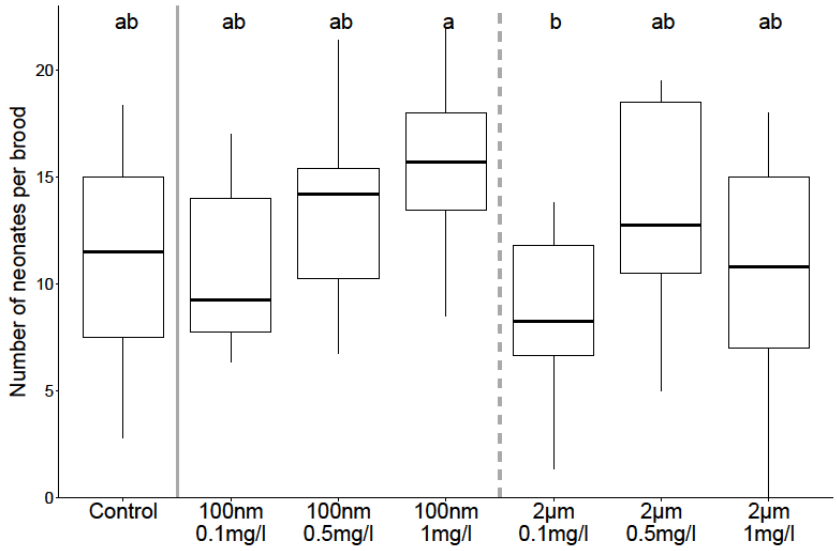

Fig. S11. Number of neonates per brood within the 21 days reproduction test, in which animals were exposed to 100 nm or 2 µm polystyrene particles at different concentrations (0.1 mg/l. 0.5 mg/l, 1 mg/l). Groups that do not share the same letter are significantly different from each other.

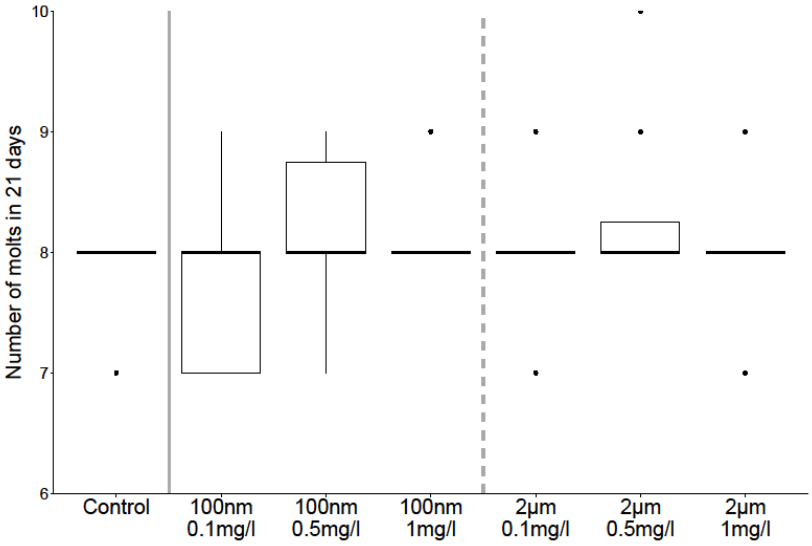

Fig. S12. Number of molts of mother animals within the 21 days reproduction test, in which animals were exposed to 100 nm or 2 µm polystyrene particles at different concentrations (0.1 mg/l. 0.5 mg/l, 1 mg/l).

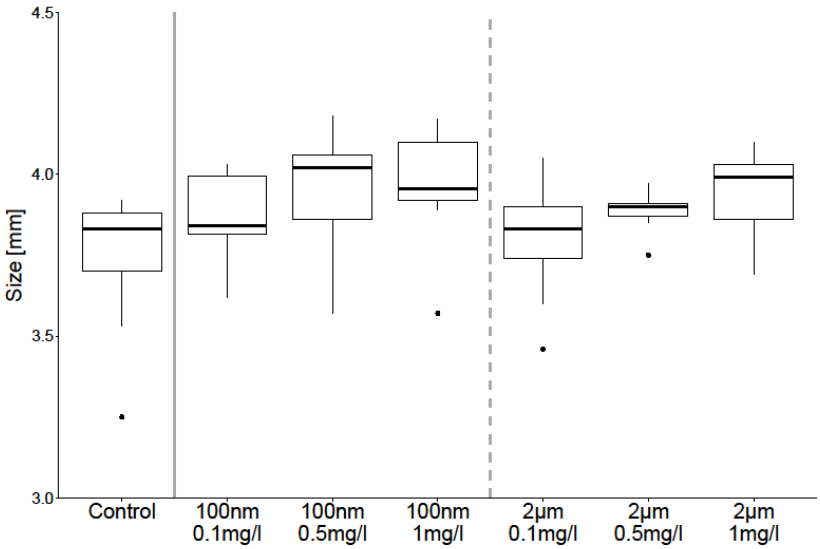

Fig. S13. Size of mother animals at the end of the 21 days reproduction test, in which animals were exposed to 100 nm or 2 µm polystyrene particles at different concentrations (0.1 mg/l. 0.5 mg/l, 1 mg/l).

Table S2

Mortality during the 21 days reproduction test, in which animals were exposed to 100 nm or 2 µm polystyrene particles at different concentrations (0.1 mg/l. 0.5 mg/l, 1 mg/l). There were 15 replicates per treatment group.

Treatment group	Number of dead animals
Control	1
100 nm, 0.1 mg/l	3
100 nm, 0.5 mg/l	2
100 nm, 1 mg/l	2
2 µm, 0.1 mg/l	2
2 µm, 0.5 mg/l	4
2 µm, 1 mg/l	3