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Significant decline of *Daphnia magna* population biomass due to microplastic exposure

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Abstract (300 words)

Even though microplastics are intensively studied, the focus of the research is mainly on relatively short term effects at high doses. Therefore there is a need to shift the focus toward more realistic, longer-term endpoints. Studies with a range of chemicals have shown that the response of populations often differs from studies in which a single organism is exposed in an individual container (as often described within standard ecotox screening assays). Here we investigate the impact of primary microplastics (1-5 µm in size) on a population of *Daphnia magna*. We first allowed a stable population of *D. magna* to develop over 29 d, after which the populations were exposed to microplastics for three weeks (concentrations ranging from 10^2 to 10^5 particles mL^{-1} and a control). We found a significant impact of microplastics on the total population of *D. magna*, with a reduction in the amount of adult daphnids. Importantly, when expressed as total biomass, exposure to 10^5 microplastics mL^{-1} resulted in a 21% reduction in total biomass compared to control. These results indicate that exposure to microplastics can result in significant adverse effects on the population of *D. magna*, including a reduction in the number of individuals as well as total biomass. Given the importance of *D. magna* in freshwater food webs, both as a grazer as well as a food source, this can potentially impact the functioning of the ecosystem.

Keywords: Daphnia magna; Carrying capacity; Microplastics; Chronic toxicity; Population dynamics

1. Introduction

There is considerable knowledge and agreement on the widespread distribution of microplastics (plastic particles <5 mm) in the environment, as well as their potential to be taken up by organisms (Auta et al., 2017; Eerkes-Medrano et al., 2015; Van Cauwenberghe et al., 2015). A recent detailed review concluded that ecological risks of microplastics are currently rare, however, if emissions continue (scenario: business as usual) risks may become widespread (SAPEA, 2019).

Over the last years the impact of microplastics on freshwater organisms has received increased attention, which is of great importance as it was understudied until recently (Dris et al., 2015; Horton et al., 2017). In most studies, the laboratory tests that assess potential adverse effects differ considerably in their outcome. For example, several studies on *D. magna* report adverse effects, including increased mortality (Aljaibachi and Callaghan, 2018; Jaikumar et al., 2018; Jemec et al., 2016), immobilization (Rehse et al., 2016), reduced feeding rates (Rist et al., 2017), growth (Martins and Guilhermino, 2018) and reduced reproductive capacity (Martins and Guilhermino, 2018; Ogonowski et al., 2016). In contrast, other studies on *D. magna* found limited or no impacts on the endpoints listed above, for example on mortality (Kokalj et al., 2018; Ogonowski et al., 2016) and reproduction (Aljaibachi and Callaghan, 2018; Imhof et al., 2017). The discrepancy between these studies calls for scientists to further investigate the potential adverse effects of microplastics to *D. magna*. Most of the laboratory studies provide *ad libitum* high quality food to *D. magna*, with some exceptions in which different food levels were included in the study. Aljaibachi and Callaghan (2018) demonstrated limited to no effects of microplastics, and related this to the selective avoidance of microplastics when there is abundant food. Jemec et al. (2016) only found increased mortality when daphnids were not fed with algae before the experiment, and no impact if they were fed. Finally, Ogonowski et al. (2016) demonstrated decreased individual growth at low algal concentrations, but not at high algal concentrations. Such effects of food quantity or quality on reduced toxicity

have been demonstrated several times before for pesticides (Alexander et al., 2013; Barmentlo et al., 2018; Ieromina et al., 2014).

The limitation of food is a common environmental aspect of bottom-up driven food webs (Hunter and Price, 1992), which can thus limit the maximum population size. The findings that microplastics can potentially reduce feeding rates (Rist et al., 2017), reproduction (Martins and Guilhermino, 2018; Ogonowski et al., 2016) and that this effect may differ with different food levels (Aljaibachi and Callaghan, 2018; Jemec et al., 2016; Ogonowski et al., 2016) give clear indications that higher organizational levels of *D. magna* could be affected as well. However, the potential impacts on higher organizational levels are heavily understudied as current studies focus mostly on the effects on the organismal or sub-organismal level (Browne et al., 2015; Rochman et al., 2016).

To study the potential effects of microplastics on higher organizational levels, we aimed to investigate the impact of microplastics on the size and structure of populations of *D. magna*. *Daphnia magna* was selected as they are relatively simple maintenance and have high reproduction rates (OECD, 2012), thus they allow for easy testing of population dynamics (van Leeuwen et al., 1987). Moreover they have an important role in the ecosystem, as grazer and as prey, and, being abundant (Forró et al., 2008). In the current study we held bottom-up driven populations of *D. magna* at food-induced carrying capacity and subsequently exposed the populations to microplastics to study effects on population size and structure. As this is a new study design, we first determined how long it takes for the populations to reach carrying capacity using different food levels, the population size at carrying capacity, and whether the populations were stable for the OECD recommended test duration of 21 d (OECD, 2012). These outcomes were subsequently used to investigate the impact of microplastics to populations of *D. magna* and the total biomass of these populations.

2. Materials and methods

2.1 *Test species and culture conditions*

Daphnia magna are small filter feeding freshwater crustaceans that have a cyclic parthogenetic reproduction, leading to populations usually dominated by female individuals (Forró et al., 2008). The population composition is dependent on stress factors like density or short day length (Eads et al., 2008). These stressors can lead to the production of males or winter eggs (ephippia) to repopulate when conditions are better (Hobaek and Larsson, 1990).

The daphnids were obtained from the longstanding culture maintained by Leiden University which is kept under similar conditions as recommended by the OECD guidelines 211 (OECD, 2012). Stock populations are held in 10-L aquaria containing 4 L of Elendt M4 medium (OECD, 2012). Cultures are kept at 22 ± 1°C, a 16-8 h day-night cycle and a pH between 6-8, and fed a diet of the algae *Pseudokirchneriella subcapitata* (10^4 cells/organism/day). Testing of the cultures every 4 months using the reference toxicant K_2CrO_7, showed that the sensitivity of the daphnids is well within the limits set by the guideline (OECD, 2004).

2.2 Microplastics

Fluoro-Max™ green fluorescent polystyrene beads with a diameter of 1 - 5 µm (mean 4.1 ± 1.0 µm) and density of 1.3 g/cc were purchased from Cospheric LLC (Goleta, CA, USA). These microplastics were brought in suspension in Elendt M4 medium, producing a stock solutions of 10^8 particles/mL. This solution was vortexed for 10 seconds to homogenize the suspension. Subsequently, for each newly prepared solution, the concentration of particles was determined by use of a hemacytometer (the average of three separate counts was used). A dilution series in Elendt M4 medium was prepared for each treatment level. Each suspension was vortexed for 10 seconds before any further use to avoid precipitation of plastics.

2.3 Experiment 1: Establishing carrying capacity

In a first experiment we determined i) how long it takes for *D. magna* to reach carrying capacity at different food levels, ii) the total amount of individuals in a population at carrying capacity,

and iii) whether the population was maintained at carrying capacity for 21 d. We followed OECD guidelines for testing of chemicals where possible during the experiment (OECD, 2012). Prior to the experiment, neonates (<24 h old) were collected and kept for 10 d. They were reared at 22 ± 1 °C, 16-8 h day-night cycle and fed tri-weekly with the algae *Pseudokirchneriella subcapitata* (10^4 cells/organism/day). At the start of the experiment (day 0), 10 daphnids were placed in 250 mL glass beakers containing 200 mL Elendt M4 medium. These daphnids were fed one of four different levels of algae concentrations, each with four replicates; 0.5, 1.0, 1.5 or 2.0 x 10^5 cells mL^{-1} day^{-1}. The beakers were randomly placed in a climate chamber and kept at 16:8h light-dark cycle, 22 ± 1 °C and a pH between 7.6 - 8.9. Aeration was provided to all beakers using silicone tubing and glass capillary pipettes to minimize any effects of the different concentrations of algae on the amount of available oxygen and the pH of the medium.

Three times each week (Mon, Wed and Fri) the daphnids were collected from the beakers; they were separated from the medium by carefully pouring the contents of a beaker through a fine meshed sieve and moved to a Petri-dish with a small amount of medium for measurements. The Petri-dish with daphnids was placed on a LED-panel (60x60cm 4000K, 3780Lm; Brightfit, Leiden, the Netherlands) and photographed (Nikon D3300, 50mm fixed focal length, shutter speed 1/320, f10, ISO 100; Nikon Company, Tokyo, Japan). The number of daphnia per beaker were then counted from the resulting images (for example, see supplement Figure S1) using Photoshop (Adobe, Inc. CC 2017).

2.4 Experiment 2: Microplastic exposure

Based on the outcomes of the carrying capacity test, we designed an experiment to test the chronic toxicity of primary microplastics on a population of daphnids at carrying capacity. Similarly as described above, 10-d old daphnids were placed in 250-mL beakers containing 200 mL of M4 medium (10 daphnids/beaker for a total of 24 beakers). We selected 1.0 x 10^5 cells mL^{-1} d^{-1} as the optimal food level for use in the microplastic exposure for three main

reasons. First, the total number of daphnids at steady state had limited variation across beakers and the population remained relatively stable (see results section 3.1 and Figure 1). Second, for pragmatic reasons the population was of a limited size and could thus be counted and measured frequently during the experiment, while any larger population size was not practically feasible. Third, given that the population could further expand exponentially with increased food levels (Figure S2) we assumed limited density related stress. Other conditions were kept equal to Experiment 1.

In the pre-exposure phase, populations were allowed to develop for 30 d. At Day 30, the exposure of the populations to microplastic was started, which lasted 21 d (comparable with OECD 211). The *D. magna* populations were exposed to control, 10^2, 10^3, 10^4 or 10^5 particles mL^{-1} (4 replicates per treatment). The selected microplastic concentrations resulted in a ration of microplastic to algal cells ranging between 1:1000 to 1:1. Every day precipitated microplastics were resuspended by careful pipetting at the bottom of every beaker. In addition, the constant aeration during the experiment resulted in movement of the water, also decreasing the amount of precipitating plastics.

Using the same procedure as described above, the populations of daphnids in each beaker were removed, photographed, placed in a new beaker with clean medium, and fed three times a week (Mon-Wed-Fri). During the exposure period, microplastics were added directly following after the daphnids were fed. The pictures were used in Photoshop to count the number of daphnids in each beaker. In addition, the size of the daphnids was determined using Photoshop. Daphnids were divided in three different size classes; adult (>2.0 mm), juvenile (1.4 – 2.0 mm) and neonate (0.7 – 1.4 mm) according to Liess et al. (2006). At the final day of the experiment, 40 adult *D. magna* (10/beaker) per treatment were randomly selected and measured from the top of their head (excluding antennae), to the base of their apical spine as described in Coors and De Meester (2008).

177 *2.5 Statistical analyses*

178 To investigate if the population size was not impacted by density stress the actual final
179 population sizes were compared with population sizes that were linearly extrapolated from the
180 lowest food level. These expected population sizes were compared with the observed
181 population sizes with a Chi-square test.

182

183 In order to investigate the possible effect of increasing concentrations of microplastics over
184 time on the daphnids, we performed linear mixed models (function *lme*, package *nlme*) with
185 replicate as the random variable to account for the repeated measures design. These models
186 were used to test for possible effects of time and microplastic concentration on the total
187 population size, total biomass and the number of adults, juveniles, neonates and ephippia.
188 Total biomass was estimated by multiplying the abundance of each life stage (neonate,
189 juvenile, adult) with their median size class (1.05, 1.70 and 3.12mm respectively). Neonate
190 and juveniles median size class were derived from the size classes as indicated by Liess et al.
191 (2006) and adult size class from the mean body length of the controls in the final population.

192

193 A possible effect of the microplastics on body length was determined using similar linear
194 models as described above, but the daphnids were nested in the respective beaker they were
195 reared in (function *lme*, package *nlme*). We tested for homogeneity of variances using
196 Levene's and for normality of the model and random variable residuals using QQ-plots. The
197 data for the number of Ephippia was square root transformed to fit these assumptions. All
198 statistical analyses were performed using R (version 3.5.0).

199

200 **3. Results**

201 *3.1 Experiment 1: Carrying capacity test*

202 The different food regimes resulted in different stable populations (Figure 1). For all four food
203 levels, population sized increased for approximately 20d after the start of the experiment. The
204 maximum population peaked at ~100 (0.5×10^5 cells mL^{-1} d^{-1}), ~250 (1.0×10^5 cells mL^{-1} d^{-1}),

~350 (x 10^5 cells mL^{-1} d^{-1}) and ~450 (2.0 x 10^5 cells mL^{-1} d^{-1}) individuals per beaker. After the initial growth, the populations leveled to a steady population of ~80 and ~120 individuals per beaker, for 0.5 x 10^5 cells mL^{-1} d^{-1} and 1.0 x 10^5 cells mL^{-1} d^{-1} respectively (Figure 1). The population for the two higher food levels were more variable over time, with ~220 and ~430 individuals per beaker, for 1.5 x 10^5 cells mL^{-1} d^{-1} and 2.0 x 10^5 cells mL^{-1} d^{-1} respectively (Figure 1).

We found that the linearly extrapolated predicted population sizes differed significantly from the observed population sizes at different food levels (Chi-squared = 12.693, df = 2, p-value = 0.0018; Fig. S2). In addition, the exponential relationship (R^2 = 0.993) showed a better fit compared to the linear relationship (dotted line; R^2 = 0.938), which indicates limited to no density related stress on the populations (Fig. S2).

3.2 Experiment 2: Microplastic exposure

Exposure to increasing concentrations of microplastics interacting with time significantly decreased the total population size (F = 4.93, p = 0.028; Figure 2A), as well as the total biomass (F = 9.90, p = 0.002; Figure 2B). The total population size decreased, dependent on time, with a maximum of 26% at the highest exposure level relative to control (Figure 2A). These changes were most pronounced for the total number of adults, which showed a dose dependent decrease after 21 d of exposure, with 38.5 ± 2.6 adult per beaker in the highest exposure and 54.3 ± 7.3 adults per beaker in the control ($F_{1,18}$ = 5.26, p = 0.034; Figure S3A). There were no clear patterns of effect for the juveniles and neonates (Figure S3B,C).

Total biomass dependent on time, was reduced up to 21% in the highest concentration relative to the control (Figure 2B). For all other treatments a reduction in biomass was also observed, but much less pronounced, with a 3%, 11%, and 9% difference, when exposed to 10^2, 10^3 and 10^4 particles mL^{-1}, respectively. This difference in total biomass can be attributed to an absolute decrease in adult daphnid abundance (Figure 3A; Figure S2A). The adult biomass after 21d of

exposure decreased from 169 ± 20 unit per beaker in control, to 120 ± 7 in the highest exposure (Figure 3A), a decrease of 29%. In the other treatments adult biomass also decreased, with a 8%, 10%, and 20% decrease when exposed to 10^2, 10^3 and 10^4 particles mL^{-1}, respectively. Importantly, the relative contribution of either the adult, juvenile or neonate biomass as percentage of the total population biomass showed no significant changes among different exposure regimes ($p > 0.05$ for all comparisons, Figure 3B). In fact, the adult daphnids contributed most to the total biomass in all different treatments (on average 63-70%) compared to juveniles or neonates.

There was no significant effect of the different treatment levels on the average length of adults after 21 d of exposure (Table 1). In addition, the total number of ephippia during the exposure period did not significantly differ among concentrations ($p > 0.05$ for both comparisons, Table 1).

4. Discussion

To date, the vast majority of studies investigating the impact of microplastics use short-term experiments, while there is much less understanding on the chronic effect of microplastics on organisms (SAPEA, 2019). In addition, in most of these studies impacts are assessed at the organismal or sub-organismal level, while there has been less focus on more ecological relevant levels of biological organization, such as populations or assemblages of organisms (Browne et al., 2015; Rochman et al., 2016). In the current study, we focused on this knowledge gap by exposing a population of *D. magna* at food-induced carrying capacity to microplastics. We observed significant impacts of microplastics on the total number of individuals in the population, as well as the biomass while the population structure remained unaffected. We acknowledge that the exposure concentrations used in our study (10^2–10^5 particles mL^{-1}) are relatively high. However, the exact concentrations of microplastics in the environment are not known, for example due to difficulties in identifying and quantifying (very small) plastics particles (SAPEA, 2019). Therefore, the environmental levels of microplastics reported in the literature are likely an underestimation of the actual environmental concentration, especially for particles in the size ranges which were used in the current study (SAPEA, 2019). And, as highlighted in the introduction, the level of microplastics in the environment will likely further increase if we continue our current level of plastic production (Huvet et al., 2016; SAPEA, 2019).

After 21 d of exposure the total biomass per beaker was reduced in all treatments, and by 21% at the highest exposure concentration compared to control. We suggest two possible explanations for this reduction in biomass. First, the accumulation of microplastics in the gut might reduce the uptake efficiency of the food, or reduce assimilation of food. After uptake microplastics can from aggregates in the gut of organisms, and as a result can cause an blockage in the gut which could reduce food uptake (Ogonowski et al., 2016). For example, exposure of the copepod *Centropages typicus* to a combination of algae and microplastics showed a significant reduction in algal feeding compared to control conditions (Cole et al.,

2013). A study by Rist et al. (2017) found a significant reduction in feeding rate, with a reduction of up to 21%. In addition, microplastics can cause intestinal alterations in organisms, as observed for the sea bass *Dicentrarchus labrax* (Pedà et al., 2016). Both examples reduce the total energy intake, which in turn reduce the energy budget available for growth and reproduction (Kooijman, 2001).

A second explanation of the reduction in biomass could be changes in the energy translocation to cope with elimination of the microplastics. For example, previous research has shown that exposure to cadmium results in molecular responses, especially in relation to growth and development, which the authors linked to an impact on somatic growth and development, and even population growth rate (Connon et al., 2008). In another study effects on maintenance were linked to effects on different levels of organization for *Caenorhabditis elegans* (Wren et al., 2011). A study on six model toxicants showed an impact of these toxicants on the cellular energy allocation, with lipid reserves being the most sensitive endpoint studied (De Coen and Janssen, 2003). Furthermore, these impacts were correlated with chronic (21 d) impacts on growth, survival, and reproduction (De Coen and Janssen, 2003).

Previous studies conducted in our laboratory used the same type of microplastic to study acute and chronic toxicity to *D. magna*, however following standardized OECD protocols (Jaikumar et al., 2018; Jaikumar et al., under review), allowing for a direct comparison among studies. Limited acute effects were observed after 96 h exposure to the same microplastics, even at concentrations up to 10^7 particles mL^{-1}. In contrast, chronic toxicity after 21 d of exposure using the standardized OECD protocol showed significant adverse effects of microplastics on the size of first brood (10^3 particles mL^{-1}), the size of the first three broods (10^2 particles mL^{-1}) and the cumulative number of neonates (10^3 particles mL^{-1}). Therefore, we expect that the reduction in total number of individuals, as well as the reduction in biomass observed in the current study to be a result of a reduction in reproductive performance, and not increased mortality. While total biomass decreased with increasing concentrations of microplastics, the

population structure was unaffected throughout exposure period as the relative distribution of adults, juveniles and neonates was never statistically different from the control. This shows that the total population decline is likely not a behavioral response by the daphnids to, for example, produce less offspring per capita. Again, this indicates that the effect is more likely hampered reproduction (Jaikumar et al., under review). Assuming food was completely consumed (but we did not measure this, and Rist et al. (2017) showed impaired feeding), this shows that there was probably energy relocation to cope with toxic stress, thus less energy available for reproductive output. In line with the principles of the Dynamic Energy Budget theory as outlined by Kooijman (Kooijman, 2001).

Ultimately, the observed reduction in population size and biomass can have knock-on effects within bottom-up controlled freshwater ecosystems, potentially resulting in a trophic cascade (Brett and Goldman, 1996; Jeppesen et al., 2011). Zooplankton play an important role in phytoplankton control, especially increasing transparency in freshwater lakes (Lampert et al., 1986). A reduction in zooplankton biomass can thus result in an increase in phytoplankton, thereby decreasing lake transparency (Jeppesen et al., 2011). In addition, zooplankton are an important food source in freshwater systems (Forró et al., 2008) for predators, and therefore changes in crustacean populations may alter the system at ecosystem level.

5. Conclusions

To conclude, this research addresses a key knowledge gap, as little is known about the ecological impacts of microplastics at higher level of biological organization (e.g. population level and assemblages) (Browne et al., 2015; Rochman et al., 2016). Most research to date has focused on (sub)organismal effects, with very limited linkages to ecological responses, such as changes in population status (e.g. biomass, population composition, and population size) (Browne et al., 2015; Rochman et al., 2016). We observed significant adverse impacts of microplastics on both the total number of individuals and total biomass of a population of *D. magna*, as well as a significant reduction in the total amount of adult daphnids. Thus,

microplastics can indeed affect the higher biological organization of bottom-up driven populations of *D. magna*. The stability of *D. magna* populations under natural conditions is important for the functioning of the freshwater ecosystem, as they are important grazers of phytoplankton, as well as a key food source for predators.

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Supplementary information:

Figure S1. Example of an image used from a *D. magna* population to determine total population size within beakers.

Figure S2. The predicted (black line) and observed (black dots) relationship between the food level (algae mL^{-1} day^{-1}) and the total number of *Daphnia magna*. The exponential relationship (striped line; $R^2 = 0.993$)) showed a better fit compared to the linear relationship (dotted line; $R^2 = 0.938$), indicating limited to no density related stress.

Figure S3. Average number (±SE, n=4) of *D. magna* A) adults, B) juveniles and C) neonates over time exposed to different concentrations of Fluoro-Max™ green fluorescent polystyrene beads (particles/mL, mean Ø = 4.1 ± 1.0µm). Continuous exposure started at t=30.

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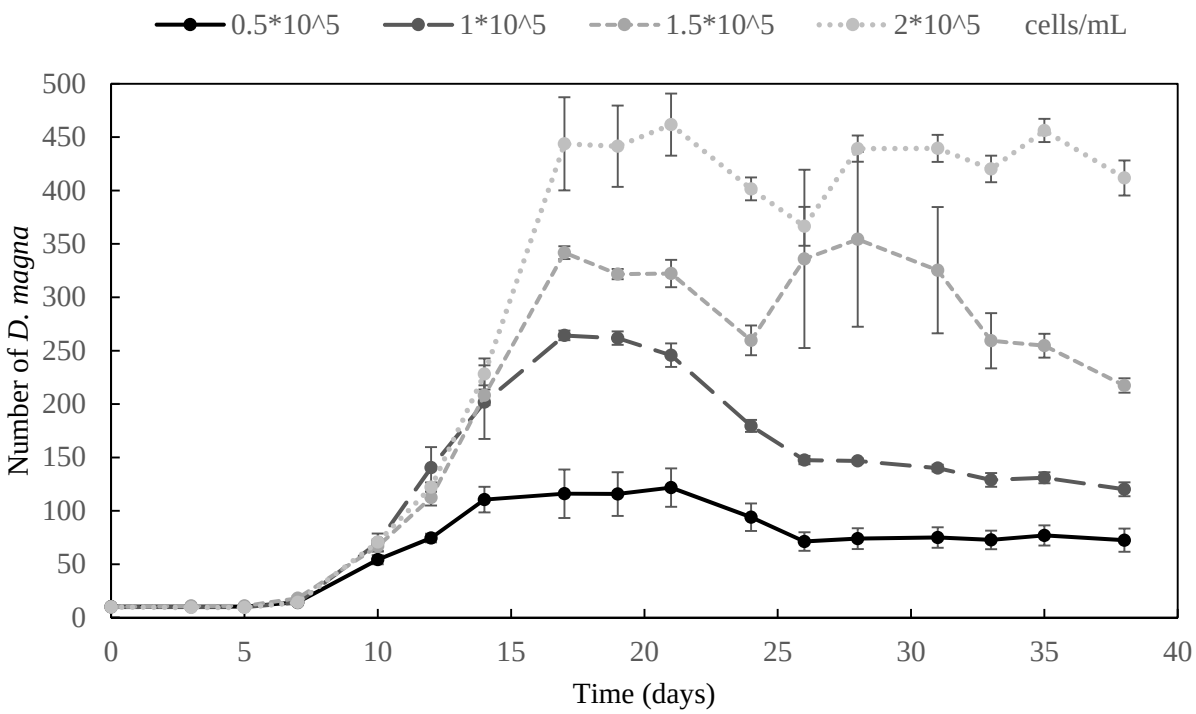


Figure 1. Average population size of *D. magna* (±SE, n=4) fed daily with different concentrations of *P. subcapitata* (cells/mL). Note that error bars are smaller than the data points in some cases.

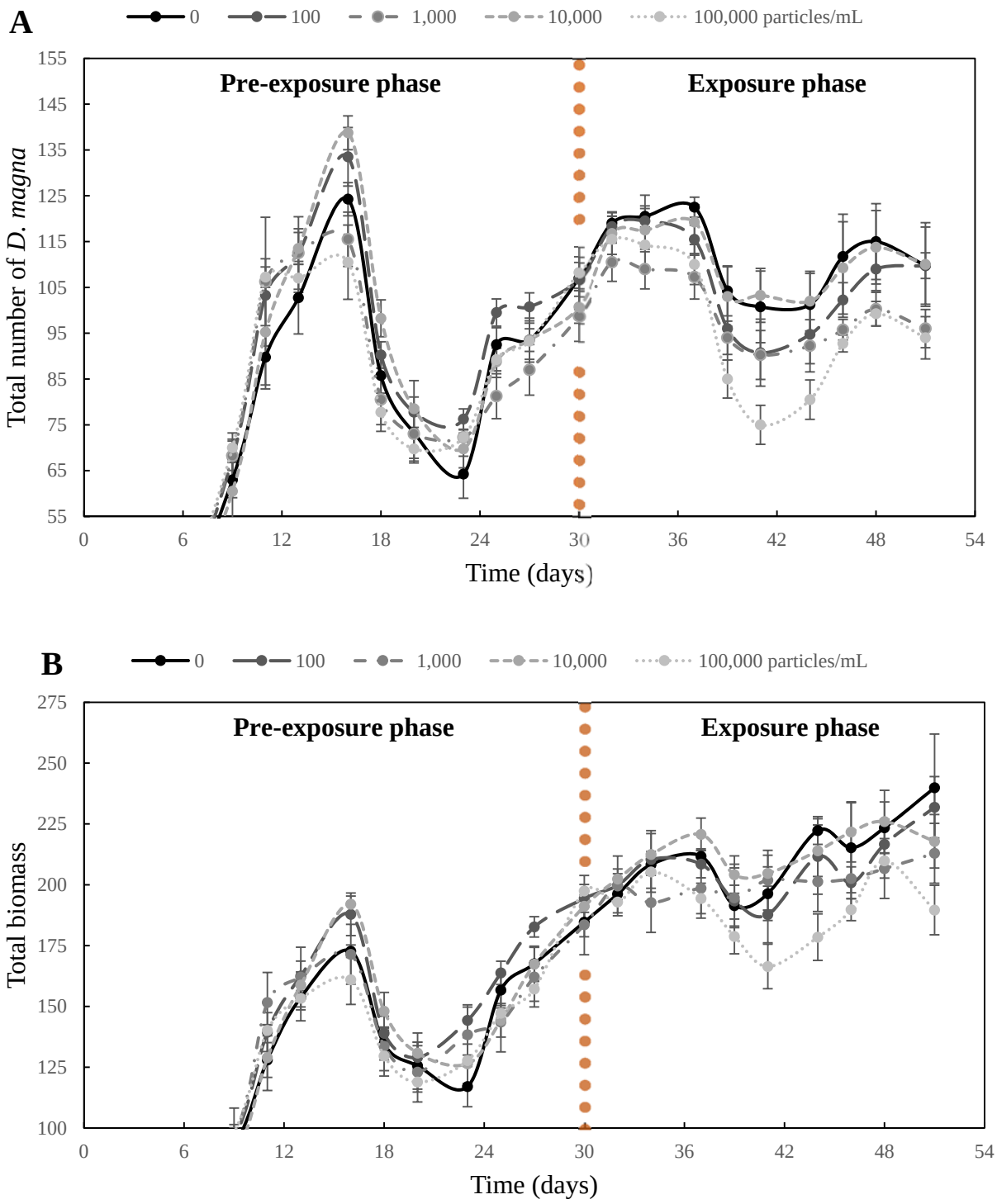
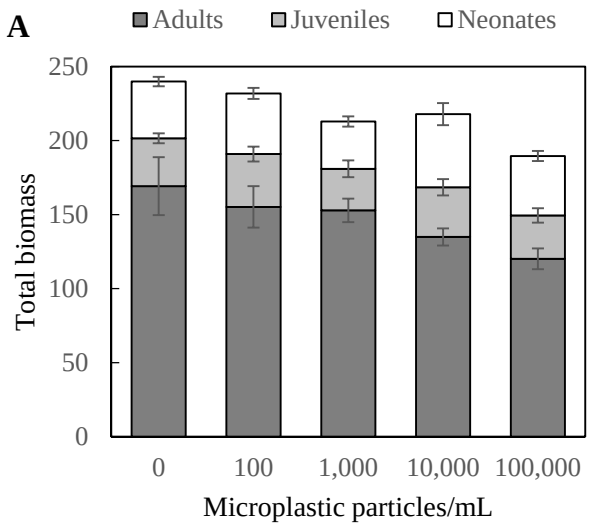


Figure 2. Average population size of *D. magna* (±SE, n=4) over time (in days) exposed to Fluoro-Max™ green fluorescent polystyrene beads (particles/mL, mean Ø = 4.1 ± 1.0µm) as a function of A) total number of individuals and B) total biomass (mean body size per life stage * abundance). Continuous exposure started at t=30. Data below 55 daphnids and a biomass of 100 are not shown for clarification purposes. Data on population dynamics of different size classes (neonate, juvenile, adult) are shown in Figure S3.



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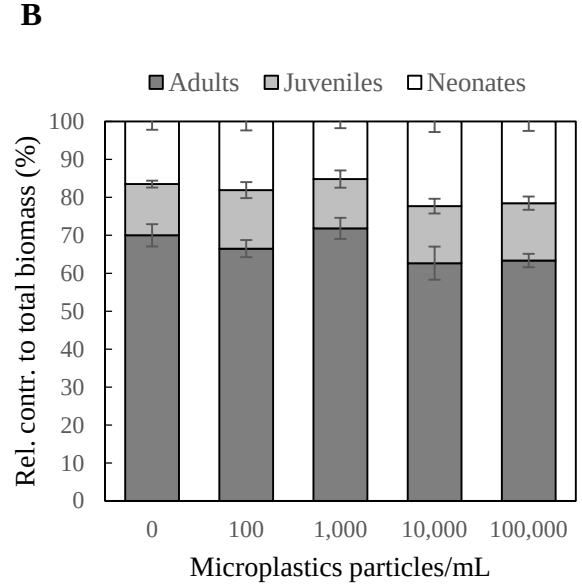

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484 **Figure 3.** Average *D. magna* population structure (±SE, n=4) per life stage (adult, juvenile,
485 neonate) after 21 days of exposure to Fluoro-Max™ green fluorescent polystyrene beads
486 (particles/mL, mean Ø = 4.1 ± 1.0µm) as function of A) total biomass (mean body size per life
487 stage * abundance) and B) relative contribution (percentage) to the total biomass.

488 **Table 1.** The average (±SE) body length of *D. magna* and number of produced ephippia after
489 21 days of exposure.

Concentration (particles mL^{-1})	Body length (mm)	Number of ephippia
0	3.12 (±0.04)	3.00 (±0.71)
100	2.98 (±0.05)	3.50 (±1.48)
1,000	2.96 (±0.05)	5.00 (±2.69)
10,000	2.89 (±0.04)	2.75 (±0.65)
100,000	2.99 (±0.05)	4.50 (±1.79)

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Supplementary Information

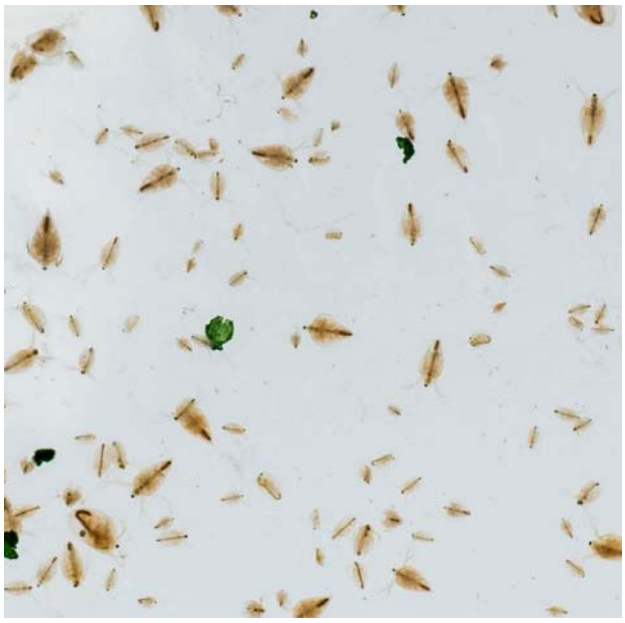


Figure S1. Example of an image used from a *D. magna* population to determine total population size within beakers.

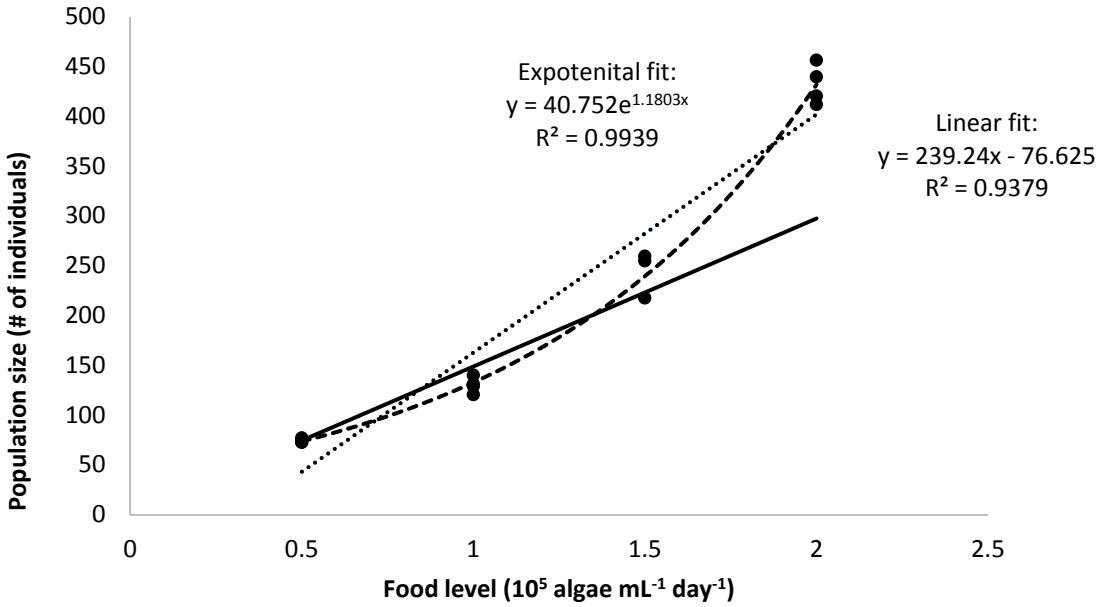
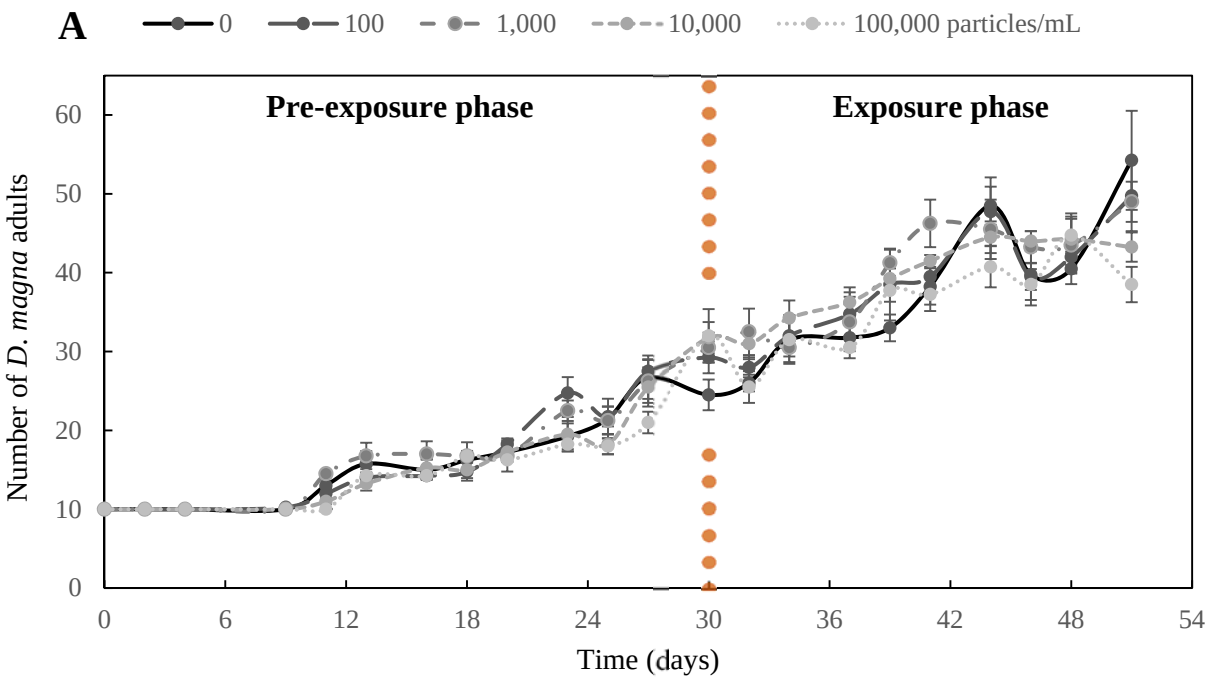
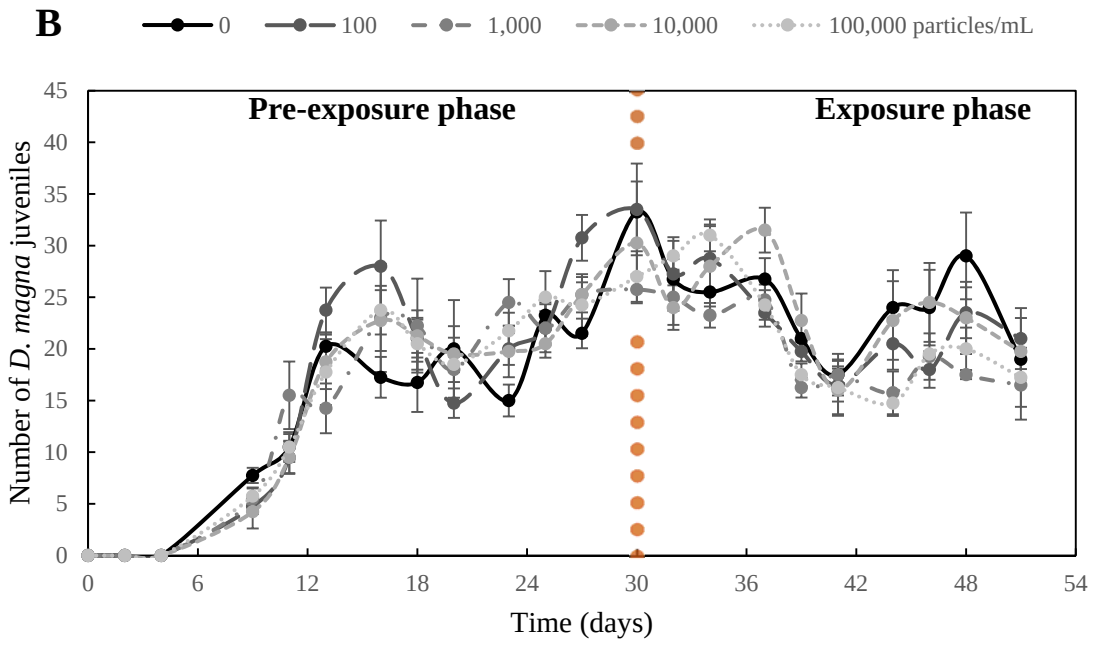


Figure S2. The predicted (black line) and observed (black dots) relationship between the food level (algae mL^{-1} day^{-1}) and the total number of *Daphnia magna*. The exponential relationship (striped line; R^2 = 0.993)) showed a better fit compared to the linear relationship (dotted line; R^2 = 0.938), indicating limited to no density related stress.


A
0
100
1,000
10,000
100,000 particles/mL
Pre-exposure phase
Exposure phase
Number of D. magna adults
Time (days)

500


B
0
100
1,000
10,000
100,000 particles/mL
Pre-exposure phase
Exposure phase
Number of D. magna juveniles
Time (days)

501

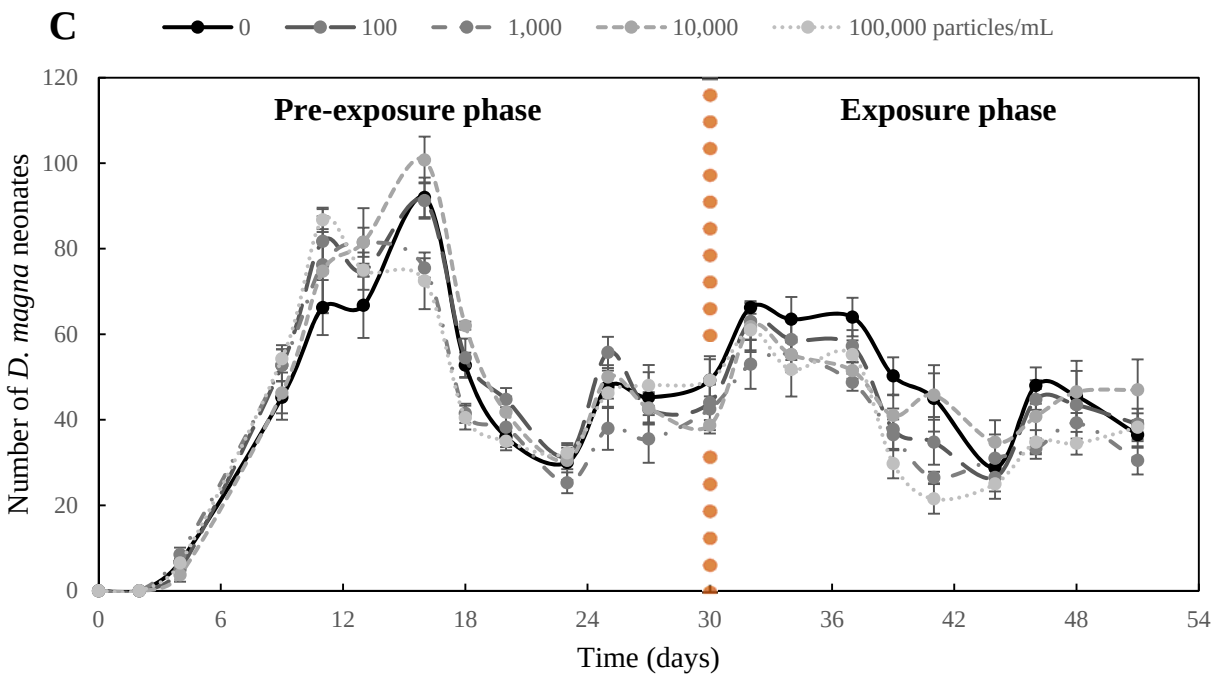


Figure S3. Average number (±SE, n=4) of *D. magna* A) adults, B) juveniles and C) neonates over time exposed to different concentrations of Fluoro-Max™ green fluorescent polystyrene beads (particles/mL, mean Ø = 4.1 ± 1.0µm). Continuous exposure started at t=30.