Microplastics but not natural particles induce multigenerational effects in *Daphnia magna*

Christoph Schür[a]*, Sebastian Zipp[a], Tobias Thalau[a], Martin Wagner[b]

[a] Department of Aquatic Ecotoxicology, Faculty of Biological Sciences, Goethe University Frankfurt am Main, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany

[b] Department of Biology, Norwegian University of Science and Technology, 5 Høgskoleringen, 7491 Trondheim, Norway

*Address correspondence to Martin Wagner, martin.wagner@ntnu.no

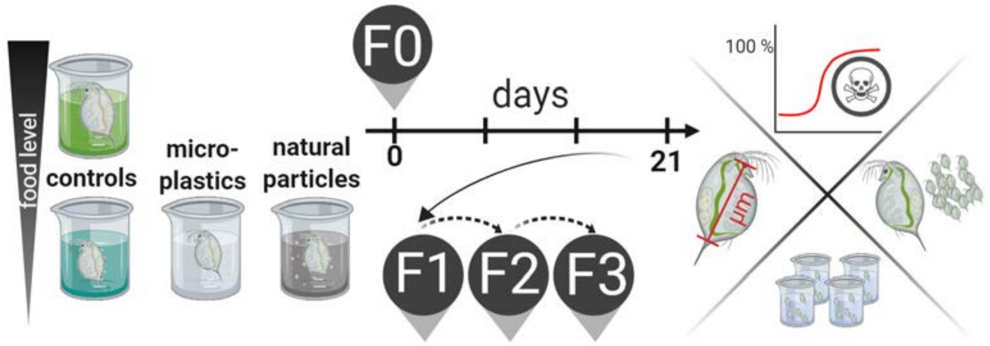
Abstract

Several studies have investigated the effects of nano- and microplastics on daphnids as key freshwater species. However, while information is abundant on the acute toxicity of plastic beads little is known regarding the multigenerational effects of irregular microplastics. In addition, a comparison of microplastics to naturally occurring particles is missing. Therefore, we investigated the effects of irregular, secondary polystyrene microplastics (< 63 µm) and kaolin as natural reference particle on the survival, reproduction, and growth of *Daphnia magna* over four generations under food-limited conditions. Additionally, we tested the sensitivity of the neonates in each generation to a reference compound as a proxy for offspring fitness. Exposure to high concentrations of microplastics (10000 and 2000 particles mL^{-1}) reduced daphnid survival, resulting in extinction within one and four generations, respectively. Microplastics also affected reproduction and growth. Importantly, an exposure to kaolin at similar concentrations did not induced negative effects. The sensitivity of neonates to potassium dichromate was not affected by maternal exposure to particles. Taken together, our study demonstrates that irregular PS particles are more toxic than natural kaolin in daphnids exposed over multiple generations under food limitation. Thus, our work builds towards more realistic exposure scenarios needed to better understand the impacts of microplastics on zooplankton.

Main findings

Microplastics increase mortality and affect life-history endpoints in *D. magna* over four generations under food limitation, while natural particles did not.

Graphical abstract



Introduction

Human activity distinctly impacts Earth system processes with potentially disastrous consequences, such as climate change and the loss of biodiversity (Rockström et al. 2009). Persson et al. (2013) and MacLeod et al. (2014) discussed the potential of chemical pollution to also compromise a “safe operating space for humanity” and defined three criteria for a pollutant to be considered a planetary boundary threat: (I) The chemical or mixture of chemicals has a disruptive effect on a vital Earth system process, (II) the disruptive effect is not discovered until it is, or inevitably will become, a problem at a planetary scale, (III) the effect of the pollutant in the environment cannot be readily reversed. The occurrence of large amounts of plastic debris in natural environments already is ubiquitous in scale (criterion II) and irreversible (III). Therefore, it is of interest to better understand its effect on earth processes, especially potential adverse effects and underlying mechanisms of plastics on biological systems at different trophic levels (Jahnke et al. 2017).

Of special concern in this context are small plastic particles formed during the degradation of larger debris, so-called secondary microplastics (MP). These can be ingested by zooplankton, which as primary consumer has a key role in aquatic food webs. Cladocera, such as Daphnia species, are model organisms for ecotoxicology and there is no shortage of studies investigating the effects of nano- and microplastics on daphnids (e.g., Ogonowski et al. (2016), Rist et al. (2017)). However, with some exceptions (e.g., Bosker et al. (2019), Jaikumar et al. (2019)), most studies report acute toxicity using high concentrations of commercially available spherical nano- and microplastics and short exposure durations. While irregular MP are predominant in the environment (Burns and Boxall 2018), the literature provides limited insight into their chronic toxicity. In addition, a multitude of other factors will modulate biota-particle interactions, such as feeding type, inter- and intra-species competition, biofilm formation, hydrodynamics, and presence of other particles (Scherer et al. 2018). Again, these are currently poorly understood.

Three other aspects are important when looking at the body of knowledge: First, short-lived biota will be exposed to MP over multiple generations and so far only one study addresses this (Martins and Guilhermino 2018). Second, both MP (Ogonowski et al. 2016; Rist et al. 2017; Scherer et al. 2017) and inorganic natural particles (Kirk 1991a) are readily ingested by daphnids without providing nutrition. Thus, it is reasonable to assume that high food levels in an experiment, such as those recommended by the OECD guideline 211 (OECD 2012), can mask potential effects occurring through food dilution or increased energy expenditure. Third, as natural particulate matter is abundant in aquatic ecosystems, the toxicity of MP needs to be compared to naturally occurring particles to investigate whether the former are indeed more toxic than the latter (Ogonowski et al. 2018; Backhaus and Wagner 2019). Here, kaolin clay as a representative for natural suspended particulate matter can adversely affect cladocerans (Kirk and Gilbert 1990; Kirk 1992; Robinson et al. 2010).

To address these aspects, we conducted a four-generation experiment in which we exposed *Daphnia magna* to irregular polystyrene MP and kaolin as a natural reference particle at three concentrations (400, 2000 and 10000 particles mL^{-1}) under food-limited conditions.

Materials & Methods

Particle preparation

Polystyrene (PS) lids of coffee-to-go cups obtained from a local bakery were used to produce irregular MP for this study. They were cut into small pieces, frozen in liquid nitrogen and then ground in a ball mill (Retsch MM400, Retsch GmbH, Germany) at 30 Hz for 30 s. The process of freezing and grinding was repeated 2–4 times to produce sufficient amounts of MP. The resulting powder was sieved to ≤ 63 µm using a sediment shaker (Retsch AS 200 basic, Retsch GmbH, Germany). While all MP used in this study was prepared in one batch, the preparation of multiple batches using identical conditions resulted in MP with similar properties (data not shown). The procedure is, thus, reproducible. Kaolin ($\sim Al_2Si_2O_5(OH)_4$, CAS 1332-58-7, Merck, Darmstadt, Germany) was sieved as described above. We characterized the density of the PS MP as described in the Supplementary materials (Table S1).

Prior to use, MP and kaolin were suspended in M4 medium (1 g L^{-1}) and shaken for 48 h. The concentrations and particle size distributions of the stock suspensions were determined using a Coulter counter (Multisizer 3, Beckman Coulter, Germany; orifice tube with 100 µm aperture diameter for a particle size range of 2.0 – 60 µm) and adjusted to the desired concentrations by dilution in M4 medium. For that, the particle suspensions were continuously stirred, 0.5-1 mL taken from the middle of the water column and transferred immediately to the Coulter counter beaker which was also stirred continuously. Scanning electron microscope images of both particle types were taken using a Hitachi S-4500 scanning electron microscope (Supplementary Material, Figure S1). The suspension was continuously stirred during transfer to the test vessels.

Daphnia culture

Ten *Daphnia magna* individuals were cultured in 1 L of Elendt M4 medium (OECD 2012) at 20 °C with a 16:8 light:dark cycle. Daphnids were fed with green algae (*Desmodesmus subspicatus*) thrice a week supplying 0.15 mg carbon per individual per day (mgC $daphnid^{-1}$ d^{-1}). The medium was completely renewed once a week.

Multigenerational study

The multigenerational study basically consisted of four consecutive semi-static reproduction experiments (21 d, OECD guideline 211) similar to the design used by Völker et al. (2013).

The decision to investigate four generations represents a compromise between maximizing the number of generations and feasibility. We used food limitation as additional stressor because we hypothesized that high food levels recommended by standard guidelines will mask the effects of a particle exposure. This accounts for the fact that food is rarely in ample supply in the real world and that the additional ingestion of particles without nutritional value will exacerbate the effect of a food limitation.

Specimens for the first generation (F0, < 24 h old neonates) were taken from the daphnid culture (see above). The offspring of the previous experiment were then transferred to the next experiment (i.e., generation) and treated identical to its parents. For this, neonates (< 24 h old) from the 3rd brood of each treatment were pooled, and 20 individuals randomly picked for the next generation (F2 animals of the PS2000 treatment produced 16 neonates, only). Each treatment consisted of 20 daphnids that were held individually in 100 mL glass beaker containing 50 mL Elendt M4 medium. Animals were fed daily with *D. subspicatus*. The medium was exchanged completely thrice a week by transferring the daphnids to new vessels. The exposure duration was 21 d for the first three generations. In the fourth generation, we extended the duration to 26 d because the reproduction in the high-food control was delayed.

The daphnids in the high-food control (HFC) without particles were fed 0.15 mgC daphnid^{-1} d^{-1} according to the OECD guideline (OECD 2012). To induce food limitation, the animals in all other treatment groups were fed a lower food level (0.05 mgC daphnid^{-1} d^{-1}). We selected this feeding regime on the basis of a pilot experiment which had shown that feeding with 0.05 mgC daphnid^{-1} d^{-1} results in a significant reduction of reproduction while still meeting the OECD validity criterion for reproductive output (Figure S2). Concurrently, survival was not affected by the low food level. The low-food treatments included another negative control group without particles (low-food control, LFC) and groups exposed to 400, 2000 and 10000 particles mL^{-1} MP or kaolin.

The particle concentrations in the exposure vessels were determined throughout the experiment with a Coulter counter using separate beakers that were prepared identically to the ones in the experiment but did not contain daphnids and algae. Parallel to the water exchanges, we collected 0.5–1 mL water from the middle of the water column from these beakers after light agitation and determined the particle concentrations using Coulter counting (see Supplementary material for details). The results indicate that the nominal and actual particle concentrations match reasonably well (Table S2). We, thus, report the results using the nominal concentrations.

Each day, we recorded the mortality of adult daphnids (immobility for 15 s after agitation, (OECD 2004)) and their reproductive output (neonates per female). Neonates were removed and pooled to create the next generation (3rd brood) or perform acute toxicity tests with potassium dichromate (4th brood). At the end of each experiment, the surviving adults were preserved in 70 % ethanol. Their size was determined using a stereo microscope (Olympus SZ61, Olympus GmbH, Germany) and the software Diskus (version 4.50.1458) by measuring the distance between the center of the eye and the base of the apical spinus (Ogonowski et al. 2016).

Neonates from the fourth brood of each generation were exposed to 0.302 to 2.5 mg L^{-1} potassium dichromate (CAS 7778-50-9, ≥ 99.0 %, Sigma-Aldrich, Steinheim, Germany) following the OECD guideline 202 for Daphnia Acute Immobilization Tests (OECD 2004). Each potassium dichromate concentration was tested in four replicates with five daphnids each. For treatments with low reproduction, we reduced the number of replicates to account for limited offspring availability (see Table S3). We derived the median lethal concentrations (LC_{50}) in each test to compare the neonates' sensitivity across treatments. Since the LC_{50} range considered valid by OECD (0.6–2.1 mg L^{-1}) is very broad, we established the baseline LC_{50} for our daphnid culture in three independent acute tests with neonates raised under culturing conditions, that is, high food levels (see Figure S3).

Data analysis

The data was analyzed using GraphPad Prism (Version 5.04 for Windows, GraphPad Software, La Jolla, California, USA) with two-way ANOVA with Bonferroni post-hoc test and RStudio 1.1.463 (RStudio Team 2016) with R Version 3.5.2 (R Core Team 2018) with the tidyverse package (Wickham 2017). Life-history parameters were analyzed using two-way ANOVA with Bonferroni multiple comparisons test of each treatment and generation against the corresponding generation in the LFC treatment group. LC_{50} values for the potassium dichromate sensitivity were estimated using linear regression (max. likelihood) in ToxRat Professional 3.0.0 (ToxRat Solutions, Alsdorf, Germany).

Results

Survival

The food limitation we used in the multigenerational study did not affect the survival of the *D. magna*. Over four generations, the survival of daphnids in the low- and high-food control groups was ≥ 80 % (Figure 1, Figure S4, Table S4). This is in accordance with the validity criterion according to the OECD guideline 211 (OECD 2012). In the treatment groups with the highest PS concentration (10000 particles mL^{-1}), mortality continuously increased after four days of exposure in the first generation (Figure 1). This resulted in an almost complete extinction of daphnids with one surviving individual after 21 d. In contrast, all animals survived when exposed to 10000 particles mL^{-1} kaolin as natural reference particle. In the treatments with medium concentrations, 79 % of animals exposed to 2000 PS particles mL^{-1} survived the first generation. The survival then declined further in the second and third generations (55 and 35 %), followed by a complete extinction in the fourth generation. In contrast, survival in the corresponding kaolin treatment was 95–100 % throughout all generations. At the lowest concentration tested (400 particles mL^{-1}), survival of animals in the PS and kaolin treatment was ≥ 75 and ≥ 90 % throughout the multigenerational study.

Reproduction

As expected, food limitation reduced the reproductive output in all treatment groups compared to the high-food control (Figure 2). The mean number of neonates per surviving female was between 29 and 39 % lower across generations in the low-food compared to the high-food control (Table S4). The reproductive output of animals from the PS groups (F0 and F1) and the kaolin groups (F0, F2, F3) was similar to the low-food control (< 5 % difference). Interestingly, we observed a marked decrease in reproduction from the first to the second generation across all groups. In the subsequent generations, the reproduction of the animals in the high and low-food control, and the kaolin treatments recovered to the initial level. In contrast, the reproductive output of daphnids exposed to PS remained > 10 % (PS4000) or > 20 % (PS2000) lower that in the corresponding low-food control. This reduction is statistically significant for animals exposed to 2000 PS particles mL^{-1} in F2 ($p < 0.05$) (two-way ANOVA with Bonferroni multiple comparisons test, generation: F (3, 351) = 37.27, treatment: F (4, 351) = 60.49, interaction: F (12, 351) = 1.714).

According to the OECD (OECD 2012), a single reproduction test with food provided at 0.1–0.2 mgC $daphnid^{-1}$ d^{-1} should yield at least 60 offspring per surviving control animal to be valid. This was achieved in the first and third generation but not in the second and fourth generation of daphnids in the high-food control. In the latter, reproduction was delayed ($p < 0.01$, Figure 1, Figure S5). Thus, we extended the 21-d period in the fourth generation up to 26 days for the HFC group in order to obtain neonates from the fourth brood for acute toxicity testing. When the fourth brood was included, F3 control animals met the validity criterion. With regard to the timing of reproduction, all other treatment groups reproduced consistently without significant differences regarding the day of the first brood (two-way ANOVA with Bonferroni multiple comparisons test: Generation: F (3, 351) = 6.798, treatment: F (4; 351) = 1.112, interaction: F (12, 351) = 4.403; Figure S5).

Growth

The level of food affected the size of adult *D. magna* after 21 d (26 d in F3 for HFC and Kaolin400) as animals from the high-food control were larger than daphnids kept at food-limited conditions in all except the fourth generation (Figure 3). The median size of animals held at low-food conditions in the first generation was 3.99 ± 0.41 mm, while the control animals held at high food levels were 4.59 ± 0.52, 4.39 ± 0.18 mm, and 4.45 ± 17 mm in the first three generations followed by a decrease to 4.04 ± 0.29 mm in the fourth generation. The animals in the HFC control group were consistently larger than the ones in the LFC control group ($p < 0.001$), except for the last generation (two-way ANOVA with Bonferroni multiple comparisons test, generation: F (3, 351) = 19.29, treatment: F (4, 351) = 45.58, interaction: F (12, 351) = 1.321). The body size of daphnids from all PS treatment groups slightly decreased over the subsequent generations. This trend was more distinct in the PS than in the kaolin treatments. This difference is statistically significant for animals exposed to 2000 PS particles mL^{-1} in generation 1 ($p < 0.001$) but not for the other treatment groups or generations.

Neonate sensitivity to potassium dichromate

We investigated the sensitivity of neonates from the fourth brood to potassium dichromate to assess offspring fitness as a result of the parents’ exposure. The tested concentrations covered the lethal concentrations for 50 % of the animals (LC_{50}) established in three experiments with neonates of parents from our laboratory culture (Figure S3). The LC_{50}s were inside that sensitivity range in the first-generation offspring from all treatment groups (Figure 4, grey areas). The acute toxicity in neonates from parents from the high-food control remained stable over the consecutive generations. In contrast, the offspring from parents that received lower food levels were more sensitive in F1 and F2. Compared to that, fourth generation neonates were less sensitive to potassium dichromate. Here, neonates from parents exposed to 2000 particles mL^{-1} kaolin had the highest LC$_{50}$.

Discussion

There is no shortage of studies that investigate MP effects on daphnids. Until autumn 2018, 14 studies were available on *D. magna*, alone (Triebskorn et al. 2018). However, the vast majority of these reports provide acute toxicity data and/or tested commercially available plastic beads. While this may be a valid point of departure for investigating the toxicity of MP, the knowledge gains are limited because acute toxicity is a result of short-term exposure to very high concentrations and spherical MP are rare in the environment. Unsurprisingly, current MP research has, thus, been criticized for its lack of “realism” (Burns and Boxall 2018).

To move forward to more relevant exposure scenarios, we investigated the multigenerational effects of irregular MP in comparison to naturally occurring kaolin particles under food-limited conditions. We consider these conditions more realistic because (1) short-lived species are usually exposed to a stressor for more than one generation, (2) plastic fragments resemble the degraded, secondary MP common in aquatic ecosystems, and (3) ample food supply is the exception not the rule. In addition, comparing the toxicity of plastic and non-plastic particles is important to investigate whether MP are indeed more toxic than the natural particulate matter ubiquitous in aquatic environments (Scherer et al. (2017), Backhaus & Wagner (2019)).

Polystyrene microplastics induce multigenerational effects in *D. magna*

Over the course of four generations, MP exposure caused overt mortality in *D. magna*, resulting in extinction during the first (10000 particles mL^{-1}) and fourth generation (2000 particles mL^{-1}). An exposure to corresponding concentrations of kaolin did not. Exposure to MP also reduced the reproduction and growth of daphnids.

So far, only one other study investigated the multigenerational effects of MP in daphnids (Martins and Guilhermino 2018). Here, *D. magna* exposed to fluorescent plastic spheres (1–5 µm, unknown polymer) at a concentration of approximately 18300 particles mL^{-1} (calculated from the information given in the paper for 2 µm beads) went extinct within two generations. This is very similar to our results, even though their experiment was conducted at a higher food level (0.322 compared to our 0.05 mgC individual^{-1} day^{-1}) and higher particle concentrations. The more severe toxicity we observed at the highest MP concentration (10000 particles mL^{-1}) suggests that either the irregular PS MP are more toxic than spherical MP and/or that food limitation increases the sensitivity of daphnids to MP. The latter hypothesis is supported by previous studies showing that food limitation amplifies the toxicity of MP in *D. magna* (Aljaibachi and Callaghan 2018). However, to quantify the impact of food limitation in our study, a full factorial design would have been required.

Investigating the population-level effects of PS microbeads (1–5 µm) in *D. magna*, Bosker et al. (2019) exposed daphnid populations at their carrying capacity (limited by food) over 21 d. The authors reported a significant decrease of the total population size and biomass. Exposure to MP concentrations similar to ours (1000 and 10000 particles mL^{-1}) reduced the number of adults but not of neonates. This is somewhat in line with our findings in such that adult survival was more affected than reproduction. Contrary to Bosker et al. (2019), we observed a decrease in daphnid size. This could be related to differences in population density which resulted in a smaller body size compared to our study. Nonetheless, the experimental approaches are hard to compare since they held a number of individuals at a population-limiting food level (food availability per individual changing throughout the population growth) while we provided a constant food amount per individual. Overall, the few available studies on multigenerational effects as well as on food limitation imply that the impacts of MP may be overlooked in standard toxicity testing, even when using a well-established model such as *D. magna*.

Natural kaolin does not affect daphnids over four generations

In contrast to PS MP, kaolin as natural reference particle did not cause multigenerational effects in our study. So far, few studies directly compare the toxicity of MP to that of naturally occurring particles. In line with our findings, Ogonowski et al. (2016) reported that exposure to irregular polyethylene MP reduced the survival and reproduction of *D. magna* whereas kaolin did not. In the shore crab *Carcinus maenas*, PS microspheres causes a transient change in oxygen consumption whereas “natural sediment” did not (Watts et al. 2016). Casado et al. (2013) and Straub et al. (2017) compared the effects of different nano- and microplastics to silica particles and generally found little to no effect of the latter both *in vitro* and *in vivo*. Thus and despite the limited evidence available, MP appear to be more toxic than natural particles.

However, suspended solids can have negative effects on aquatic biota (Bilotta and Brazier 2008) irrespective of their natural or anthropogenic origin. The former are, thus, not necessarily more benign than the latter. Accordingly, a range of studies reported effects of kaolin on the feeding rate, growth, reproduction, and population dynamics in daphnid species

(Kirk and Gilbert 1990; Kirk 1991b; Kirk 1991a; Kirk 1992; Robinson et al. 2010; Maisanaba et al. 2015). However, suspended solids in surface waters are diverse regarding their physicochemical properties. Thus, one major challenge is to find appropriate reference materials matching the properties of MP as close as possible (Scherer et al. 2018).

Why are microplastics more toxic than kaolin?

The observation that PS MP are more toxic than kaolin can be explained by differences in their physicochemical properties. Regarding their irregular shape and surface structure, both particles are very similar (Figure S1). However, kaolin particles are smaller than PS particles, with the major size fraction being < 10 µm in diameter (Figure S6). Compared to that, the size distribution of PS particles is more evenly spread between 2 and 50 µm. Another obvious difference is that kaolin (2.6 g cm^{-3}) is much denser than PS (0.96–1.05 g cm^{-3}). Accordingly, the higher toxicity of PS MP in our study might be related to its larger size and lower density. However, although not verified experimentally in this study, there is a range of other relevant properties, most importantly the surface charge and chemical composition.

While differences in size and density may be directly linked to the adverse effects of PS MP in our study, they will also dictate the exposure of the animal to the particles. Particle size is a relevant factor that determines the particle uptake by aquatic biota (Scherer et al. 2018). *D. magna* readily ingests MP < 90 µm (Scherer et al. 2017). The morphology of the filter apparatus determines the lower size limit (200 nm; Brendelberger (1991)). Additionally, processes like drinking and rectal water uptake can lead to the ingestion of very small particles (Smirnov 2017). Thus, the major size fraction of both particle types would be ingestible by *D. magna*. More importantly, the denser kaolin has a higher sinking velocity than PS. This results in a rapid sedimentation of kaolin while most PS particles remained buoyant (Table S5). Thus, the PS exposure was rather continuous while each medium exchange led to a pulsed exposure to kaolin. As daphnids mainly feed from the water column, PS might have been more available than kaolin. Therefore, we hypothesize that differences in particle behavior resulted in different exposures that in turn affected the outcomes we observed. To test this, uptake studies would be needed which are complicated by the lack of analytical techniques to quantify very small, non-fluorescent particles in biota.

There are multiple ways to account for particle fate in MP studies. To compensate for differences in sedimentation, continuous agitation can be used to keep particles in suspension irrespective of density and shape (Frydkjær et al. 2017; Gerdes et al. 2018). However, particle exposure in natural systems will always depend on the properties of the suspended solids. Daphnids will move in the water column along food gradients (Neary et al. 1994) and, thus, also feed on sedimented material. Simply characterizing the particle fate in the water column of the system, therefore, will not fully cover the interaction of particles with the animal. Therefore, future toxicity studies should quantify the fraction that is available to the animals (e.g., concentrations in the water column) as well as the uptake of MP and reference particles to enable a better comparison of their effects.

While investigating the chemicals leaching from the MP used here is beyond the scope of our study, they may contribute to the observed toxicity. Recently, Zimmermann et al. (2019) demonstrated that one out of four PS consumer products contain chemicals inducing *in vitro* toxicity. As this included baseline toxicity in the Microtox assay which is correlated with adverse effects in *D. magna* (Calleja et al. 1986), it is possible that compounds leaching from the MP in our study had an effect. In contrast, Lithner et al. (2009) did not observe acute toxicity when exposing *D. magna* to leachates from a disposable PS drinking cup. The same was true for *Nitocra spinipes* exposed to leachates from UV radiated PS drinking cups (Bejgarn et al. (2015). Regardless these differences, PS will leach, amongst others, its monomer styrene which is toxic in daphnids at high concentrations (Cushman et al. 1997). Accordingly, the contribution of chemicals leaching from PS MP remains to be investigated in future work.

Potential mechanisms

The underlying idea of our multigenerational study is that the effect of a treatment propagates from parents to offspring and, thus, exacerbates over generations. We challenged daphnids by limiting their food supply and expected this to reduce the fitness of the neonates forming consecutive generations (Tessier and Consolatti 1989). This is because daphnids supply their offspring with energy reserves in relation to their own energy reserve (Tessier et al. 1983).

We observed a decline of reproduction, body size and an increase of neonate sensitivity to potassium dichromate from the first to the second generation of *D. magna* held under food-limited conditions. This is an indicator for the potential depletion of maternal energy reserves, since the animals in the first generation were derived from a culture held at higher food concentrations. In addition, a change in population density might have contributed as well as the parents of the first generation were held at 10 individuals L^{-1} whereas their offspring were held at 0.05 individuals L^{-1} during the experiments. In any case, the multigenerational effects of the food limitation are probably mediated via a reduced energy transfer from mothers to offspring.

Generally, a hormetic response and phenotypic selection could have taken place as well. The results generally question the suitability of potassium dichromate to assess offspring fitness, since we did not observe a correlation between LC_{50} values and other endpoints. Pieters and Liess (2006) for example observed changes in fenvalerate sensitivity of offspring to adults held at different food levels. Therefore, other chemicals might be a better indicator for effects produced by particle exposure under food limitation. This could be a promising field for future studies to explore as this generally appears to be a sensible approach.

The effects of the food limitation on reproduction and growth in our study were exacerbated by the exposure to PS MP. A reduction in feeding rate, food dilution and increased rejection behavior can decrease the intake of nutrients. Daphnids reduce feeding upon exposure to both natural particles (Kirk 1991a) as well as MP (Ogonowski et al. 2016; Rist et al. 2017). Additionally, non-nutritious particles increase rejection behavior and, thus, further decrease the food intake (Kirk 1991a). Importantly, food dilution through non-nutritious particles will also decrease the energy intake at the same energy expenditure. Particle exposures can also increase the energetic cost caused by additional cleaning and rejection behavior (Richman and Dodson 1983). Apart from these energetic effects, MP might affect the physiology of the digestive system. Unfortunately, little is known about the digestive processes in daphnids (Smirnov 2017).

Taken together, there are a number of plausible mechanisms by which MP ingestion can affect the energy budget of daphnids. However, because direct toxicity will also affect the energy intake and expenditure (e.g., for detoxication) it remains to be demonstrated which mechanism is dominant for MP.

Conclusions

In this multigenerational study, we exposed *D. magna* over four generations to PS MP and kaolin as a natural reference particle. Importantly, daphnids were held at food-limited conditions which reduced reproduction and growth but did not affect survival. MP negatively affected all life-history endpoint except time to maturation, while kaolin did not. The toxicity of MP was most pronounced regarding the daphnids' survival, increased over the generations and resulted in the extinction of animals in the first (10000 particles mL^{-1}) and fourth generation (2000 particles mL^{-1}). This highlights that MP have multigenerational effects probably caused by a decrease of maternal fitness and nutritional status. The absence of toxicity of kaolin can at least partially be attributed to a lower bioavailability resulting from sedimentation. While it remains challenging to find appropriate reference particles that closely match the physicochemical properties of MP, our study demonstrates that irregular PS particles are more toxic than natural kaolin. Thus, our work builds towards more realistic exposure scenarios that cover irregular MP and natural reference materials as well as food limitation and multigenerational effects.

Author contributions

CS and MW conceived the study and designed the study; CS, TT, and SZ conducted the experiments; CS analyzed the data; CS and MW interpreted the data and wrote the manuscript; all authors commented on the manuscript.

Declaration of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the German Federal Ministry for Education and Research [02WRS1378I]. The authors thank Niklas Döring (Goethe University) for his assistance with the scanning electron microscope and Lisa Zimmermann (Goethe University) and Lucian Iordachescu (Aalborg University) for assistance with FTIR analysis. The graphical abstract was created with BioRender.

Supplemental Data

The supplemental materials are available ###.

References

Aljaibachi R, Callaghan A. 2018. Impact of polystyrene microplastics on *Daphnia magna* mortality and reproduction in relation to food availability. PeerJ. 6:e4601. doi:10.7717/peerj.4601.

Backhaus T, Wagner M. 2019. Microplastics in the Environment: Much Ado about Nothing? A Debate. Glob Chall.:1900022. doi:10.1002/gch2.201900022.

Bejgarn S, MacLeod M, Bogdal C, Breitholtz M. 2015. Toxicity of leachate from weathering plastics: An exploratory screening study with *Nitocra spinipes*. Chemosphere. 132:114–119. doi:10.1016/j.chemosphere.2015.03.010.

Bilotta GS, Brazier RE. 2008. Understanding the influence of suspended solids on water quality and aquatic biota. Water Res. 42(12):2849–2861. doi:10.1016/j.watres.2008.03.018.

Bosker T, Olthof G, Vijver MG, Baas J, Barmentlo SH. 2019. Significant decline of *Daphnia magna* population biomass due to microplastic exposure. Environ Pollut. doi:10.1016/j.envpol.2019.04.067. https://linkinghub.elsevier.com/retrieve/pii/S0269749119304191.

Brendelberger H. 1991. Filter mesh size of cladocerans predicts retention efficiency for bacteria. Limnol Oceanogr. 36(5):884–894. doi:10.4319/lo.1991.36.5.0884.

Burns EE, Boxall ABA. 2018. Microplastics in the aquatic environment: Evidence for or against adverse impacts and major knowledge gaps: Microplastics in the environment. Environ Toxicol Chem. doi:10.1002/etc.4268. http://doi.wiley.com/10.1002/etc.4268.

Calleja A, Baldasano JM, Mulet A. 1986. Toxicity analysis of leachates from hazardous wastes via microtox and *Daphnia magna*. Toxic Assess. 1(1):73–83. doi:10.1002/tox.2540010107.

Casado MP, Macken A, Byrne HJ. 2013. Ecotoxicological assessment of silica and polystyrene nanoparticles assessed by a multitrophic test battery. Environ Int. 51(Supplement C):97–105. doi:10.1016/j.envint.2012.11.001.

Cushman JR, Rausina GA, Cruzan G, Gilbert J, Williams E, Harrass MC, Sousa JV, Putt AE, Garvey NA, St. Laurent JP, et al. 1997. Ecotoxicity hazard assessment of styrene. Ecotoxicol Environ Saf. 37(2):173–180. doi:10.1006/eesa.1997.1540.

Frydkjær CK, Iversen N, Roslev P. 2017. Ingestion and egestion of microplastics by the cladoceran *Daphnia magna*: Effects of regular and irregular shaped plastic and sorbed phenanthrene. Bull Environ Contam Toxicol. doi:10.1007/s00128-017-2186-3. http://link.springer.com/10.1007/s00128-017-2186-3.

Gerdes Z, Hermann M, Ogonowski M, Gorokhova E. 2018. A serial dilution method for assessment of microplastic toxicity in suspension.

Jahnke A, Arp HPH, Escher BI, Gewert B, Gorokhova E, Kühnel D, Ogonowski M, Potthoff A, Rummel C, Schmitt-Jansen M, et al. 2017. Reducing uncertainty and confronting ignorance about the possible impacts of weathering plastic in the marine environment. Environ Sci Technol Lett. 4(3):85–90. doi:10.1021/acs.estlett.7b00008.

Jaikumar G, Brun NR, Vijver MG, Bosker T. 2019. Reproductive toxicity of primary and secondary microplastics to three cladocerans during chronic exposure. Environ Pollut. doi:10.1016/j.envpol.2019.03.085. https://linkinghub.elsevier.com/retrieve/pii/S026974911834781X.

Kirk KL. 1991a. Suspended clay reduces *Daphnia* feeding rate: behavioural mechanisms. Freshw Biol. 25(2):357–365. doi:10.1111/j.1365-2427.1991.tb00498.x.

Kirk KL. 1991b. Inorganic particles alter competition in grazing plankton: The role of selective feeding. Ecology. 72(3):915–923. doi:10.2307/1940593.

Kirk KL. 1992. Effects of suspended clay on *Daphnia* body growth and fitness. Freshw Biol. 28(1):103–109. doi:10.1111/j.1365-2427.1992.tb00566.x.

Kirk KL, Gilbert JJ. 1990. Suspended clay and the population dynamics of planktonic rotifers and cladocerans. Ecology. 71(5):1741–1755. doi:10.2307/1937582.

Lambert S, Wagner M. 2018. Microplastics are contaminants of emerging concern in freshwater environments: An overview. In: Freshwater Microplastics. Springer, Cham. (The Handbook of Environmental Chemistry). p. 1–23. https://link.springer.com/chapter/10.1007/978-3-319-61615-5_1.

Lithner D, Damberg J, Dave G, Larsson Å. 2009. Leachates from plastic consumer products – Screening for toxicity with *Daphnia magna*. Chemosphere. 74(9):1195–1200. doi:10.1016/j.chemosphere.2008.11.022.

MacLeod M, Breitholtz M, Cousins IT, Wit CA de, Persson LM, Rudén C, McLachlan MS. 2014. Identifying chemicals that are planetary boundary threats. Environ Sci Technol. 48(19):11057–11063. doi:10.1021/es501893m.

Maisanaba S, Pichardo S, Puerto M, Gutiérrez-Praena D, Cameán AM, Jos A. 2015. Toxicological evaluation of clay minerals and derived nanocomposites: A review. Environ Res. 138:233–254. doi:10.1016/j.envres.2014.12.024.

Martins A, Guilhermino L. 2018. Transgenerational effects and recovery of microplastics exposure in model populations of the freshwater cladoceran *Daphnia magna* Straus. Sci Total Environ. 631–632:421–428. doi:10.1016/j.scitotenv.2018.03.054.

Neary J, Cash K, McCauley E. 1994. Behavioural aggregation of *Daphnia pulex* in response to food gradients.

OECD. 2004. Test no. 202: *Daphnia sp.* acute immobilisation test. Paris: Organisation for Economic Co-operation and Development. http://www.oecd-ilibrary.org/content/book/9789264069947-en.

OECD. 2012. Test no. 211: *Daphnia magna* reproduction test. Paris: Organisation for Economic Co-operation and Development. http://www.oecd-ilibrary.org/content/book/9789264185203-en.

Ogonowski M, Gerdes Z, Gorokhova E. 2018. What we know and what we think we know about microplastic effects – A critical perspective. Curr Opin Environ Sci Health. 1:41–46. doi:10.1016/j.coesh.2017.09.001.

Ogonowski M, Schür C, Jarsén Å, Gorokhova E. 2016. The effects of natural and anthropogenic microparticles on individual fitness in *Daphnia magna*. PLOS ONE. 11(5):e0155063. doi:10.1371/journal.pone.0155063.

Persson LM, Breitholtz M, Cousins IT, de Wit CA, MacLeod M, McLachlan MS. 2013. Confronting unknown planetary boundary threats from chemical pollution. Environ Sci Technol. 47(22):12619–12622. doi:10.1021/es402501c.

Pieters BJ, Liess M. 2006. Maternal nutritional state determines the sensitivity of *Daphnia magna* offspring to short-term Fenvalerate exposure. Aquat Toxicol. 76(3):268–277. doi:10.1016/j.aquatox.2005.09.013.

R Core Team. 2018. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.

Richman S, Dodson SI. 1983. The effect of food quality on feeding and respiration by *Daphnia* and *Diaptomus*. Limnol Oceanogr. 28(5):948–956. doi:10.4319/lo.1983.28.5.0948.

Rist S, Baun A, Hartmann NB. 2017. Ingestion of micro- and nanoplastics in *Daphnia magna* – Quantification of body burdens and assessment of feeding rates and reproduction. Environ Pollut. 228:398–407. doi:10.1016/j.envpol.2017.05.048.

Robinson SE, Capper NA, Klaine SJ. 2010. The effects of continuous and pulsed exposures of suspended clay on the survival, growth, and reproduction of *Daphnia magna*. Environ Toxicol Chem. 29(1):168–175. doi:10.1002/etc.4.

Rockström J, Steffen W, Noone K, Persson Å, Chapin FS, Lambin EF, Lenton TM, Scheffer M, Folke C, Schellnhuber HJ, et al. 2009. A safe operating space for humanity. nature. 461(7263):472–475.

RStudio Team. 2016. RStudio: Integrated development environment for R. Boston, MA: RStudio, Inc. http://www.rstudio.com/.

Scherer C, Brennholt N, Reifferscheid G, Wagner M. 2017. Feeding type and development drive the ingestion of microplastics by freshwater invertebrates. Sci Rep. 7(1). doi:10.1038/s41598-017-17191-7. http://www.nature.com/articles/s41598-017-17191-7.

Scherer C, Weber A, Lambert S, Wagner M. 2018. Interactions of microplastics with freshwater biota. In: Freshwater Microplastics. Springer, Cham. (The Handbook of Environmental Chemistry). p. 153–180. https://link.springer.com/chapter/10.1007/978-3-319-61615-5_8.

Smirnov NN. 2017. Physiology of the Cladocera. Second edition. London, United Kingdom ; San Diego, CA, United States: Elsevier/AP, Academic Press, an imprint of Elsevier.

Straub S, Hirsch PE, Burkhardt-Holm P. 2017. Biodegradable and petroleum-based microplastics do not differ in their ingestion and excretion but in their biological effects in a freshwater invertebrate *Gammarus fossarum*. Int J Environ Res Public Health. 14(7):774. doi:10.3390/ijerph14070774.

Tessier AJ, Consolatti NL. 1989. Variation in offspring size in Daphnia and consequences for individual fitness. Oikos. 56(2):269. doi:10.2307/3565347.

Tessier AJ, Henry LL, Goulden CE, Durand MW. 1983. Starvation in *Daphnia*: energy reserves and reproductive allocation. Limnol Oceanogr. 28(4):667–676.

Triebskorn R, Braunbeck T, Grummt T, Hanslik L, Huppertsberg S, Jekel M, Knepper TP, Krais S, Müller YK, Pittroff M, et al. 2018. Relevance of nano- and microplastics for freshwater ecosystems: A critical review. TrAC Trends Anal Chem. doi:10.1016/j.trac.2018.11.023. https://linkinghub.elsevier.com/retrieve/pii/S0165993618305272.

Völker C, Boedicker C, Daubenthaler J, Oetken M, Oehlmann J. 2013. Comparative toxicity assessment of nanosilver on three *Daphnia* species in acute, chronic and multi-generation experiments. Shankar SS, editor. PLoS ONE. 8(10). doi:10.1371/journal.pone.0075026. http://dx.plos.org/10.1371/journal.pone.0075026.

Watts AJR, Urbina MA, Goodhead R, Moger J, Lewis C, Galloway TS. 2016. Effect of microplastic on the gills of the shore crab *Carcinus maenas*. Environ Sci Technol. 50(10):5364–5369. doi:10.1021/acs.est.6b01187.

Wickham H. 2017. Tidyverse: Easily install and load the “Tidyverse.” https://CRAN.R-project.org/package=tidyverse.

Zimmermann L, Dierkes G, Ternes TA, Völker C, Wagner M. 2019. Benchmarking the in vitro toxicity and chemical composition of plastic consumer products. Environ Sci Technol. 53(19):11467–11477. doi:10.1021/acs.est.9b02293.

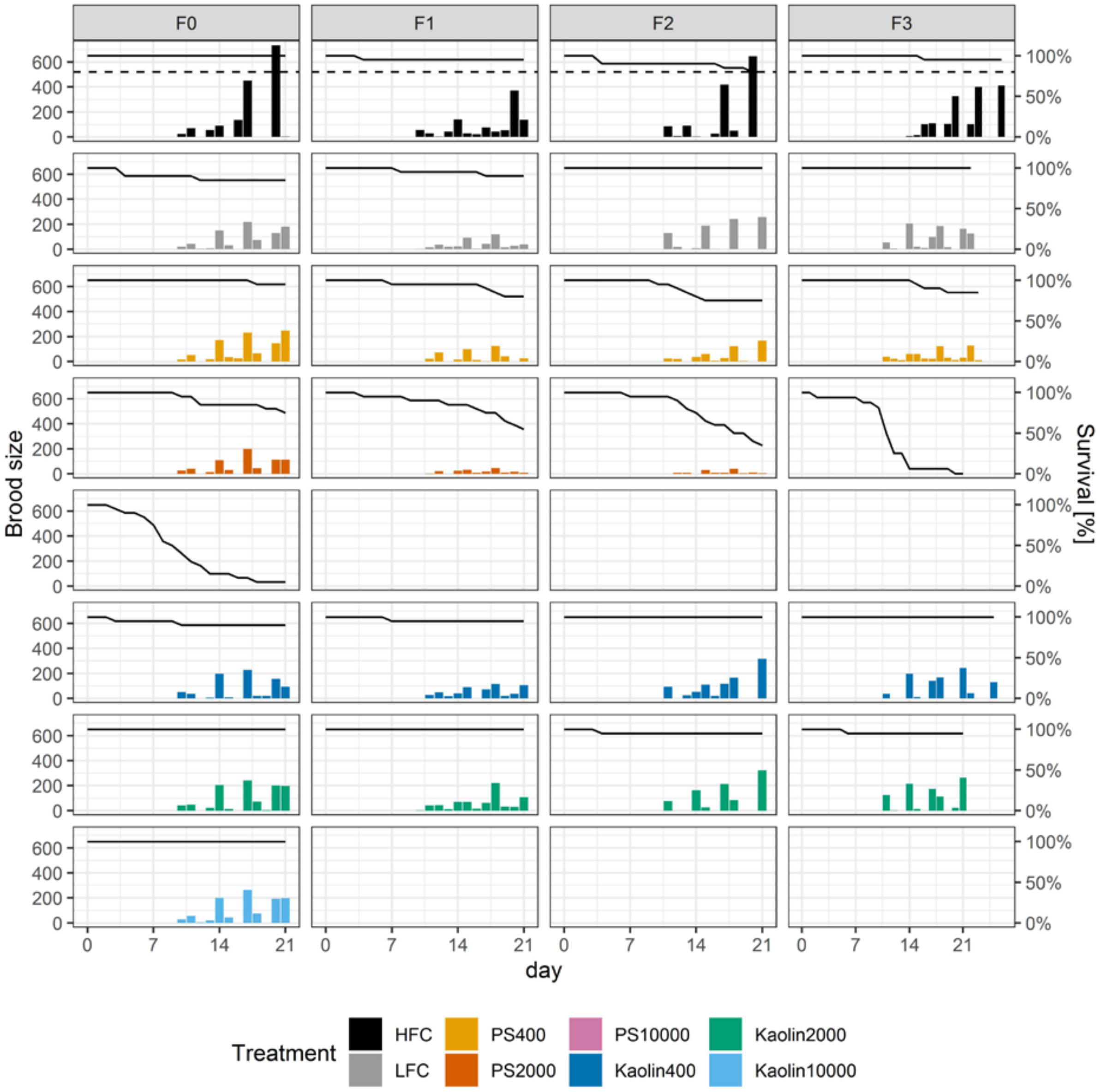


Figure 1: Survival and reproduction of *D. magna* exposed to polystyrene (PS) microplastics and kaolin over the course of four generations. The animals were exposed to 400, 2000 and 10000 particles mL^{-1} of PS microplastics or kaolin. Relative survival data (black lines) is plotted on the right axis, total reproductive output per day (bars) is plotted on the left axis. HFC = high-food control, LFC = low-food control. The treatment groups PS10000 (extinct) and Kaolin10000 were discontinued after the first generation (F0).

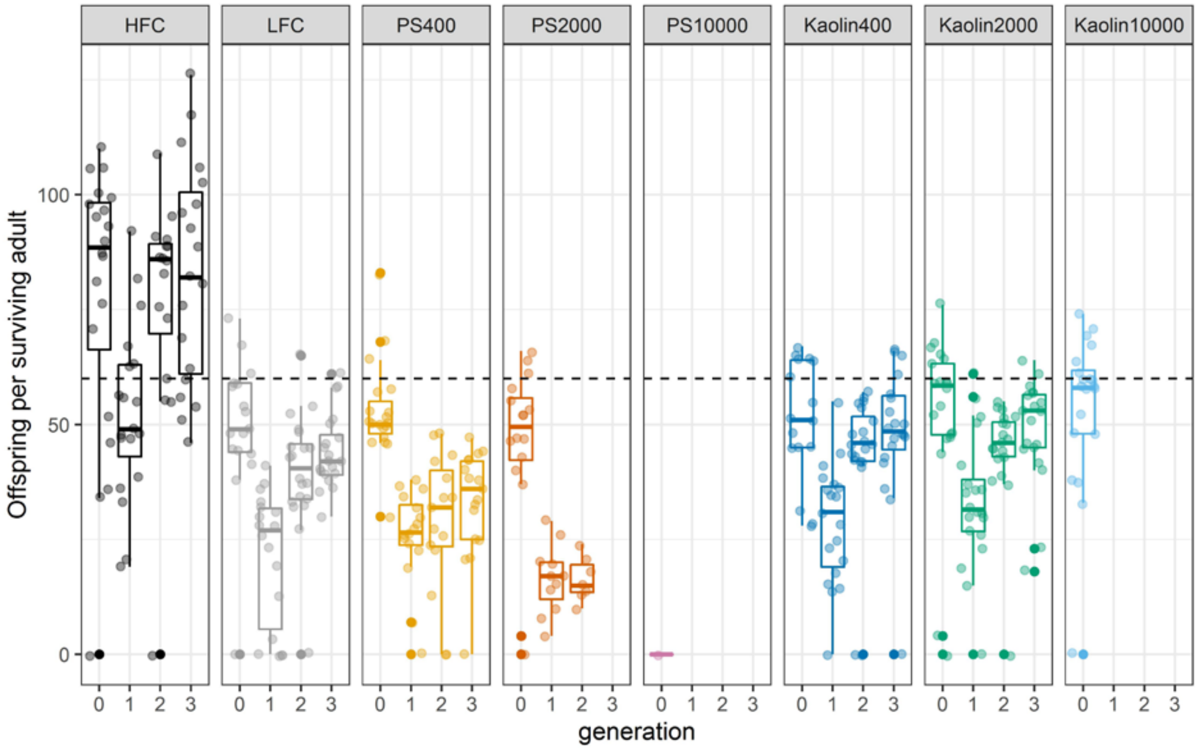


Figure 2: Offspring produced per surviving *D. magna* exposed to polystyrene (PS) microplastics and kaolin over the course of four generations. The animals were exposed to 400, 2000 and 10000 particles mL^{-1} of PS microplastics or kaolin. HFC = high-food control, LFC = low-food control. The treatment groups PS10000 (extinct) and Kaolin10000 were discontinued after the first generation (F0).

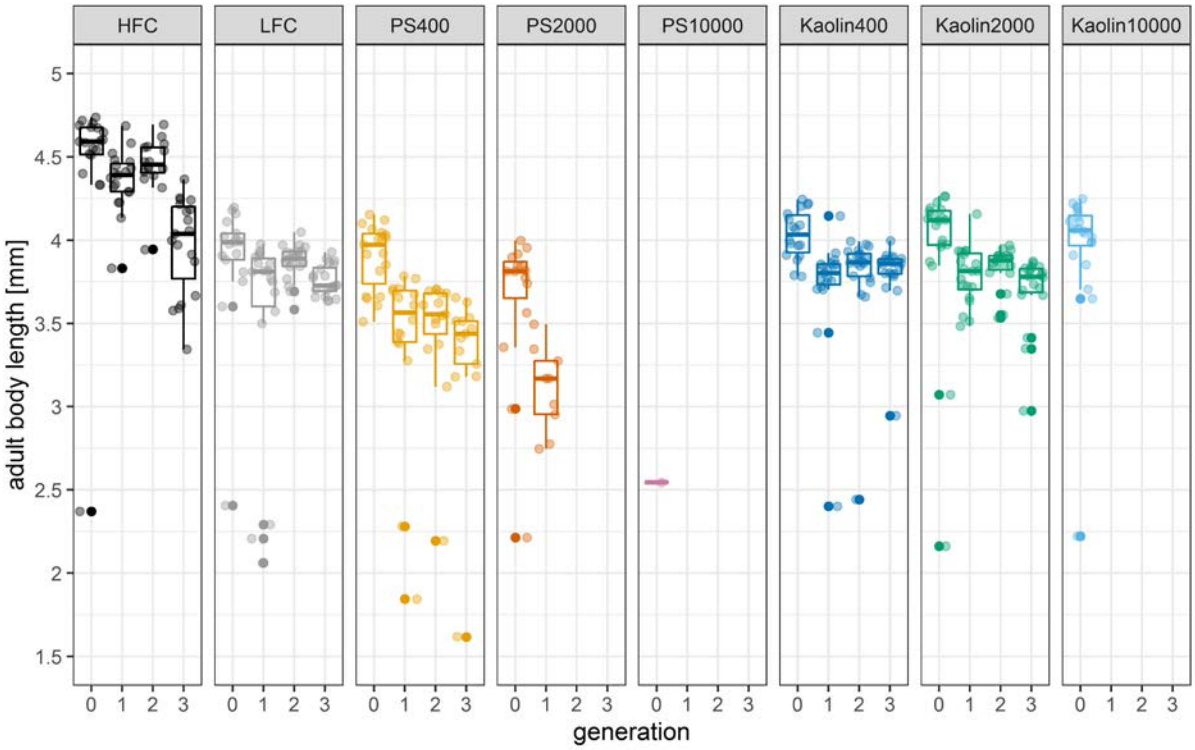


Figure 3: Size of adult *D. magna* individuals exposed to polystyrene (PS) microplastics and kaolin over the course of four generations. The animals were exposed to 400, 2000 and 10000 particles mL^{-1} of PS microplastics or kaolin. HFC = high-food control, LFC = low-food control. The treatment groups PS10000 (extinct) and Kaolin10000 were discontinued after the first generation (F0). The size was determined at the end of each generation.

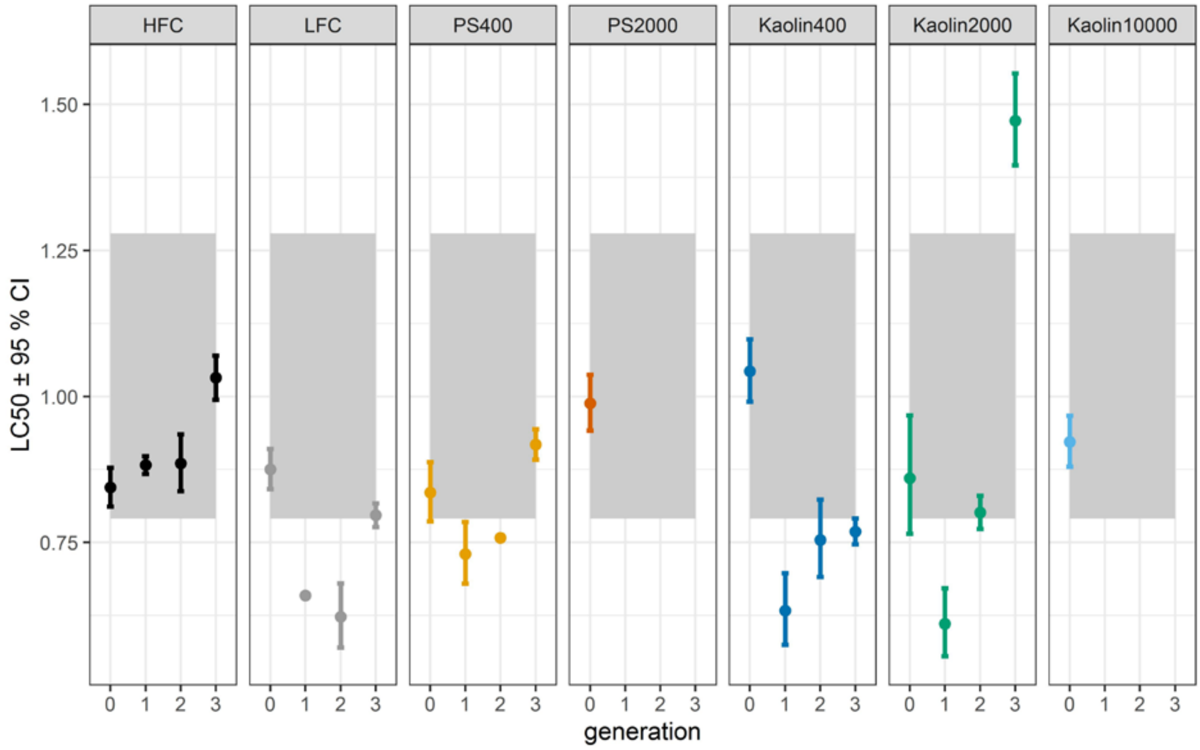


Figure 4: Acute toxicity of potassium dichromate (LC_{50}) in offspring of *D. magna* exposed to polystyrene (PS) microplastics and kaolin over the course of four generations. HFC = high-food control, LFC = low-food control. Data is missing in treatment groups which did not produce a sufficient number of neonates.