Incubation in Wastewater Reduces the Multigenerational Effects of Microplastics in *Daphnia magna*

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**ABSTRACT:** The aging of microplastics in the environment changes their physicochemical properties. While this may affect their toxicity, comparative data on the effects of aged compared to pristine microplastics are scarce. One of those aging processes is the sorption of chemicals, which has mainly been studied for individual pollutants present in marine ecosystems. To investigate how the sorption of a complex mixture of freshwater pollutants affects the toxicity of microplastics, we incubated irregular polystyrene particles (≤63 μm) in either wastewater or ultrapure water. We exposed *Daphnia magna* to these aged microplastics and their pristine counterparts (80, 400, 2000, and 10,000 particles mL−1) over four generations using food limitation as an additional, environmentally realistic stressor. Both particle types affect the survival, reproduction, adult and neonate body lengths, and growth. An exposure to pristine microplastics results in the extinction of the third generation of daphnids. In contrast, wastewater-incubated particles induced a lower mortality. The incubation with wastewater does not change the microplastics’ size, surface charge, and structure. Consistent with the literature, we assume that the adsorption of dissolved organic matter is a key aging process reducing the toxicity of microplastics. Consequently, toxicity testing using pristine microplastics may overestimate the effects of plastic particles in nature.

**INTRODUCTION**

Small plastic particles (microplastics, MPs) are ubiquitous in the aquatic environment,^1^ where they can interact with and affect a large number of biota.^2^ MPs undergo transformation processes in the environment driven by chemical, physical, and biological processes.^3^ This “aging” greatly affects the behavior and fate of MPs in the aquatic environment. Nonetheless, many ecotoxicological studies investigate the effects of pristine and/or spherical MPs, even though they are not very representative of MPs in the environment. Previous research indicates that aging and the presence of natural organic matter alter the toxicity of engineered nanoparticles.^[4,5]^ It is currently unclear how this applies to MPs, but it is likely that aging also modulates the MP toxicity.

MPs may undergo a number of different aging processes that can affect their behavior and fate in the environment: biofilm formation can alter the particle density and surface chemistry,^6^ and chemicals can adsorb or absorb to the particles that then may act as vectors and increase the chemical exposure of biota (“Trojan horse” or “vector effect”).^[7]^ Likewise, natural organic matter is abundant in the aquatic environment and can also adsorb to plastic particles, forming a corona, and alter their surface chemistry and behavior.^[8]^ All these processes will probably affect MP–biota interactions but are currently largely unaccounted for in the ecotoxicological research.

Previous studies have relatively consistently shown that particle toxicity changes in the presence of or after treatment with dissolved organic matter,^[9,10]^ humic acids,^[11]^ and wastewater.^[5,12]^ The latter is a relevant scenario because even though wastewater treatment plants effectively remove MP, they discharge large amounts of effluents that can constitute a significant fraction of the water in smaller water bodies.^[13]^ Whether and how the adsorption of the complex chemical mixture present in wastewater affects the toxicity of MPs remains unclear to date. In addition, previous studies cover the acute toxicity of aged spherical microplastics, only. Thus, the chronic, long-term effects of an exposure to pristine versus aged, irregular MPs commonly found in nature remain unknown.

Accordingly, the aim of this study is to compare the long-term effects of irregular polystyrene MPs after incubation in either filtered raw wastewater or ultrapure water on *Daphnia magna* over four generations. This multigenerational setup allows investigating effects beyond a single daphnid lifecycle.

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Moreover, the experiment was conducted under food limitation because this more closely mimics environmental conditions. We used this experimental design in a previous study and demonstrated that MPs are more toxic than natural particles. This approach was combined with experiments monitoring the individual growth of daphnids in relation to their maternal food availability and MP exposure. We hypothesized that the toxicity of wastewater-incubated MP increases in case the sorption of wastewater-borne pollutants dominates (vector effect). Alternatively, we assume the toxicity to decrease if the adsorption of dissolved organic matter or biofilm formation is the driving factor.

# Materials and Methods

**Particle Preparation and Characterization.** We obtained polystyrene (PS) coffee-to-go-cup lids from a local bakery to produce the MPs as described previously. In brief, the lids were cut into small pieces, frozen in liquid nitrogen, and then ground in a swing mill (Retsch MM400, Retsch, Germany). The resulting powder was sieved to ≤0.63 µm (Retsch AS200 basic, Retsch, Germany) and characterized as described in Schürr et al.

The PS MPs we used in this study were either incubated in wastewater (wastewater-incubated, wwMP) or in ultrapure water (pristine, MP). To produce the former, we sampled the influent of the wastewater treatment plant Bad Homburg vor der Höhe (size class IV, Germany). The influent is raw wastewater that is treated with a bar screen, only. The 24 h composite sample consisted of 1 L of wastewater sampled every 2 h between March 31st and April 1st, 2019, 8 am (pH: 7.4; electric conductivity: 934 µS cm⁻¹). No rainfall was recorded in the 5 days prior to sampling.

The wastewater was filtered using a 0.2 µm filter (Rapid Flow system, Nalgene) directly after sampling to remove suspended solids and microorganisms. The MP was then incubated in the filtrate at 1 g L⁻¹ for 38 h at 4 °C. After that, we recovered the incubated MP by vacuum filtration using a 0.2 µm filter. The particles were then frozen at −80 °C and lyophilized to remove the residual water (Alpha 1−4 LSC plus, Martin Christ, Germany). The pristine MPs were treated identically except that they were incubated in ultrapure water instead of wastewater. Both particle types were stored at −80 °C to minimize the degradation of potentially sorbed chemicals and the particles themselves.

The concentrations and particle size distributions (2−60 µm, Figure S1) of the stock suspensions were determined after a Coulter counter (100 µm aperture, Multizizer 3, Beckman Coulter, Germany; measurements in filtrated (<0.2 µm) 0.98% NaCl solution, electric conductivity:17.03 mS cm⁻¹; pH: 7.36). The correlation between the nominal and measured exposure concentrations via this method is described in Schürr et al. The zeta potentials of the particles (Figure S2) were determined after suspension in M4 medium using a Zetasizer Nano ZS (red laser, 4 mW, 632.8 nm). Both particle types were imaged using scanning electron microscopy (SEM) after freeze-drying (Figure 1). For that, 20 µL of each suspension was transferred to the sample holder, dried under a heat lamp, sputtered with gold, and imaged in a Hitachi S-4500 system. We performed an experiment to investigate the biofilm formation and changes in the surface structure of the particles over 9 days in M4 medium (conductivity: 703 µS cm⁻¹; pH: 6.94) using SEM (for details, see Supporting Information and Figure S5). The Fourier transform infrared spectroscopy (ATR-FTIR) spectra (FTIR Spectrum Two, PerkinElmer; LiTa03 detector, range: 4000−450 cm⁻¹) of the raw material before and after grading and sieving and the two particle powders after incubation and freeze-drying (MP and wwMP) are given in the Supporting Information (Figures S3 and S4). The spectral data is available at figshare under doi 10.6084/m9.figshare.12311495. Additional information on the material and particle characterization can be found in Schürr et al.

**Daphnia Culture.** Ten *D. magna* individuals were cultured in 1 L of Elendt M4 medium at 20 °C with a 16:8 h light:dark cycle. The daphnids were fed with the algae *Desmodesmus subspicatus* thrice a week at 0.2 mg carbon per individual per day (mgC daphnid⁻¹ d⁻¹). The medium was fully renewed once a week.

**Multigenerational Experiment.** The multigenerational experiment basically consisted of four consecutive semi-static reproduction experiments (21 days, OECD guideline 221), similar to the design used in Schürr et al. Each generation included two control groups held at different food levels and treatments with four concentrations per particle type (pristine MP and wastewater-incubated wwMP). The specimens for the first generation (F0, <24 h old neonates) were taken from the daphnid culture (see above). The offspring of this experiment was transferred to the next experiment (i.e., generation) and treated identical to its parents. For this, <24 h old neonates from the third brood of each treatment were pooled, and 20 individuals were randomly picked for the next generation with the exception of animals of the MP10000 group (seven neonates in F1 and 16 neonates in F2 constituted the following generations for this treatment group).

The animals in each treatment were held individually in a 100 mL glass beaker containing 50 mL of Elendt M4 medium that was fully exchanged thrice weekly by transferring the parent animal to a new vessel. Animals were fed daily with *D. subspicatus* with daphnids in the high food control (HFC, negative control without MPs) receiving 0.2 mgC daphnid⁻¹ d⁻¹ according to the Organisation for Economic Co-operation and Development (OECD) guideline. The animals in all other treatment groups were fed a lower food level of 0.05 mgC daphnid⁻¹ d⁻¹ to induce food limitation that decreases the reproduction but not survival. The low-food treatments included another negative control group without particles (low food control, LFC).

![Figure 1](https://dx.doi.org/10.1021/acs.est.0c07911)
The daphnids were exposed to 80, 400, 2000, and 10,000 particles mL$^{-1}$ of pristine or wastewater-incubated MPs over the course of four generations. Each week, we prepared a fresh stock suspension by suspending the respective MP powder in M4 medium for 48 h on an orbital shaker. The stock suspensions were then transferred to new test vessels with each of the three weekly water exchanges (resulting in a total use period of 9 days), and parent animals were carefully added using a pipette.

We recorded the mortality (15 s immobility after agitation$^{16}$) and their reproductive output (neonates per female) daily. The neonates were removed and discarded (first and second broods), pooled to create the next generation (third brood), or transferred to 70% ethanol for the size determination (fourth brood). The parent animals were photographed at the end of each generation to determine their length (center of the eye to the base of the apical spinus).

**Growth Experiments.** To investigate the effects of the maternal diet on the growth curve during the MP exposure, we conducted two experiments in which we exposed daphnids over 21 days to MPs and wwMPs and monitored their individual growth thrice a week. The first experiment was carried out with 10 neonates per group taken from the Daphnia culture and is therefore equivalent to the F0 generation of the multigenerational experiment. For the second experiment, a separate culture was reared at low food levels (0.05 mgC individual$^{-1}$ day$^{-1}$) for 16 days (equivalent to F0 in the multigenerational experiment). Neonates (<24 h old) from these two cultures were exposed to MPs as described above. The number of replicates was increased to 15 and 20 in the treatment groups exposed to 2000 and 10,000 particles mL$^{-1}$, respectively, to account for the high mortality in the prior experiments. During each water exchange (thrice weekly, on days 0, 2, 5, 7, 9, 12, 14, 16, 19, and 21), each individual’s body length was measured from the center of the eye to the base of the apical spinus.

**Data Analysis.** The data analysis was carried out using R 3.6.1$^{17}$ with RStudio 1.2.1335$^{18}$ and the tidyverse package version 1.2.1.$^{19}$ The survival data were analyzed using Fisher’s exact tests in R. All other data were analyzed using two-way ANOVA with Bonferroni multiple comparison tests in GraphPad Prism (version 5.04 for Windows, GraphPad Software, La Jolla, California, USA). The treatments were compared against the LFC group from the corresponding generation. The growth data were fitted using a von Bertalanffy growth function with bootstrapped confidence intervals according to Ogle$^{20}$ using the R packages FSA$^{21}$ and car.$^{22}$ The details, code, and parameters are provided in the Supporting Information. The raw data and model outputs are available at figshare under doi 10.6084/m9.figshare.12311495.

Boxplots were created with the geom_boxplot() function of ggplot2$^{23}$ and followed the basic boxplot of McGill (1978).$^{24}$ Additional R packages used for the analysis and visualization include readxl$^{25}$ and patchwork.$^{26}$

The MP10000 treatment was excluded from statistical analyses because the low survival, reproduction, and extinction in F2 resulted in a small sample size. The animals that died throughout the experiment were counted toward mortality but not toward the other endpoints. The animals that died from handling were completely excluded from all the analyses.

**RESULTS**

**Particle Characterization.** The size distributions of the pristine and wastewater-incubated MPs are very similar (Figure S1). The mean zeta potentials of the particles incubated in ultrapure water and wastewater are $-10.02 \pm 0.93$ and $-10.96 \pm 2.09$ mV, respectively ($n = 10$ each, Figure S2). The SEM images of the MPs after lyophilization show no obvious differences in the surface morphology (Figure 1). Likewise, the surface morphology is not altered by incubation in M4 medium, and there is no apparent biofilm formation over the
maximum time the MP suspensions were used in the experiments (9 and 2 days of suspension on an orbital shaker followed by a 7 day use period, Figure S6). The FTIR spectra are similar for the two MP types (Figures S3 and S4). The particle behavior after the application in the exposure vessels differed between the particle types: soon after mixing of the stock suspensions in M4 medium, the pristine MPs either floated or sedimented to a larger degree than the wastewater-incubated MPs that remained in the water column more consistently (Figure S5).

Survival. The survival of daphnids was affected by the exposure to MPs in concert with food limitation, but not by food limitation alone (Figure 2). The mortality was <20% in the control groups except in the F1 of either particle type was lower than that of the control groups. Interestingly, the survival in these treatment groups increased over the generations, and mortality was lower in the animals exposed to wastewater-incubated particles.

Both MP types had a concentration-dependent impact on survival in F0–F2 but not in F3 (Figure 2, Table S1). The daphnids exposed to 80 particles mL\(^{-1}\) of either particle type were unaffected with a maximum mortality of 25% in the fourth generation of the MP80 treatment group (\(p > 0.05\)). The survival of animals exposed to 400 and 2000 MP mL\(^{-1}\) (MP400/MP2000) decreased from F0 to F1 and increased to approximately 80% in the consecutive generations. Only two animals survived in the F1 generation of the MP2000 treatment (\(p < 0.001\) compared to F1 of the LFC group), so this can be considered a bottleneck event. However, these two animals produced sufficient offsprings to start F2. Only 5% of \(D.\) magna survived when exposed to 10,000 MP mL\(^{-1}\) (MP10000, \(p < 0.001\)) in the first generation. They produced only seven neonates forming F1 followed by the extinction of that treatment group in F2 (\(p < 0.05\)).

Reproduction. The limitation of food supply and the exposure to MPs affected the reproduction of \(D.\) magna (Figure S7, Table S1). The mean numbers of offsprings per surviving adult in the first generation were 155 ± 48.6 and 54.2 ± 8.79 in the HFC and LFC, respectively. Again, the experiment was valid according to the OECD validity criterion for reproduction (>60 neonates per surviving adult in the HFC\(^{15}\)). The daphnids in the HFC group produced significantly more offsprings over the course of each generation compared to the LFC group (\(p < 0.001\), two-way ANOVA with Bonferroni multiple comparison tests, generation: F (3, 663) = 84, treatment: F (8, 663) = 314, interaction: F (24, 663) = 12.74).

The reproductive output of daphnids from all the treatment groups decreased from the first to the second generation (Figure 3). For the HFC, this trend continued for F1 (130.2 ± 19.0 neonates per surviving adult) and F2 (84.3 ± 15.2) followed by a slight increase to 88.3 ± 26.1 neonates per surviving adult in F3. Under food limitation, the mean reproductive output remained below 60 for all other treatments (LFC, MP/wwMP80–10,000). The reproduction was significantly lower compared to the LFC of the
Figure 4. Body length of an adult _D. magna_ exposed to pristine microplastics (MP) and wastewater-incubated microplastics (wwMP) over the four generations. The animals were held at high food levels (high food control, HFC), low food levels (low food control, LFC), and low food levels in combination with 80, 400, 2000, and 10,000 MP or wwMP mL$^{-1}$. The MP10000 treatment group went extinct in F2 (crosses). Two-way ANOVA with Bonferroni multiple comparison tests against the corresponding LFC (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

Figure 5. Body length development of daphnids originating from parents fed with high (A + C) or low (B + D) food levels. The offspring was unexposed (black and gray, HFC/LFC) or exposed to polystyrene microplastics (80, 400, 2000, and 10,000 particles mL$^{-1}$) incubated in wastewater (wwMP, C + D, green) and ultrapure water (MP, A + B, blue) prior to exposure.
corresponding generation for the daphnids from the treatments MP2000 (F1 and F3: \( p < 0.01 \)), wwMP2000 (F1–F3: \( p < 0.01 \)), and wwMP10000 (F0–F3: \( p < 0.001 \)).

An exposure to MP affected the timing of reproduction in the third and the fourth generations. In comparison to the animals from the LFC from the corresponding generation, the day of the first reproduction (Figures S8 and S9) was significantly delayed in the daphnids exposed to pristine MPs (MP2000, F2 and F3: \( p < 0.05 \)). The MP10000 treatment was excluded from the statistical analysis due to few data points and subsequent extinction. Compared to the LFC, the reproduction was delayed in all treatment groups with wastewater-incubated MPs (wwMP80–10,000, F2 and F3: \( p < 0.05 \)) with the exception of wwMP2000 in the F3 generation.

**Body Length.** The reduced food supply and MP exposure significantly affected the body length of adults after 21 days (Figure 4, Table S1). The adult daphnids were significantly larger in the HFC than in the corresponding LFC (\( p < 0.001 \), two-way ANOVA with Bonferroni multiple comparison tests, generation: \( F (3, 663) = 36.23 \), treatment: \( F (8, 663) = 104.4 \), interaction: \( F (24, 663) = 2.04 \)). The *D. magna* species exposed to pristine and wastewater-incubated MPs at concentrations of 2000 particles mL\(^{-1} \) or higher were significantly smaller compared to the animals from the corresponding LFC (\( p < 0.05 \)).

The body length of the neonates of the fourth brood was also affected by food limitation and an exposure to MP (see detailed results in the Supporting Information, Figure S10 and Table S2).

**Growth Curve Experiments.** As a follow up, we conducted two experiments to investigate the body length development of individuals held at conditions similar to the multigenerational study with high and low maternal food levels. This is representative for the daphnids in F0 (parents from the high food culture) and F1–F3 of the multigenerational experiment (parents from the low food level F0).

For the animals in the control groups provided with high amounts of food, the food status of the parental generation played no role with regard to the length development (Figure S11). Both growth curves mostly overlap. In the daphnids with low maternal food levels receiving low food levels, we observed a higher initial growth but a similar terminal length after 21 days compared to animals originating from a high food culture. This indicates an immediate effect of maternal food limitation coupled with food limitation on the growth. The daphnids fed with high food levels grew very similarly independent of whether their parents had received high or low food quantities. In contrast, animals fed low food levels grew faster when their parents had been starved compared to the offspring of parents that had received high food levels. After 21 days, the maternal food status did not affect the length of animals from the HFC or LFC.

The animals with high maternal food levels that were exposed to 10,000 particles mL\(^{-1} \) pristine MPs had an observable effect on the length development (Figure 5). However, we observed a high mortality in this group that caused the poor data coverage beyond day 10. This effect was less pronounced for the daphnids exposed to the same concentration of wastewater-incubated MPs. The lower concentrations produced no apparent effects on the length development notwithstanding the particle type.

When we exposed individuals originating from a culture with low maternal food levels to MPs, the length development was reduced in a concentration-dependent manner for both particle types starting from 2000 particles mL\(^{-1} \). Similar to the LFC group originating from the low-food culture, we observed a higher initial growth preceding a longer phase of low growth after this initial spurt. Overall, low maternal food status coupled with food limitation of the offspring led to a higher initial growth, while high food availability led to a higher growth irrespective of maternal food status.

## DISCUSSION

We compared the multigenerational effects of irregular MPs that were incubated in wastewater or ultrapure water over the four generations of *D. magna* held under food limitation. We found that the exposure to wastewater-incubated MPs resulted in a lower mortality than pristine MPs. The toxicity of the two particle types did not differ significantly for other life history parameters.

**Multigenerational Effects.** In a previous study, we used a similar design to compare the effects of PS MPs to natural clay particles.\(^{14} \) We demonstrated that MPs affected the life history of *D. magna* with increasing effects over the generations while kaolin did not. A number of general patterns are consistent across the two studies, most notably the change in median body size from the first to the second generation, which we hypothesized to be due to the changes in the population density. Although the PS MP treatment group in Schür et al. (2020) is very similar to the one with the pristine MP used here, we observed differences between both studies. For example, the daphnids exposed to 10,000 MP mL\(^{-1} \) went extinct in F0 in the previous and in F1 in the present study. The animals exposed to 2000 MP particles mL\(^{-1} \) did not survive F3,\(^{14} \) whereas in this study, they recovered from the high mortality in F0 and F1 and survived throughout all the four generations. Overall, the lower toxicity observed in the present study could be due to the removal of the particle fraction <0.2 μm and of chemicals by filtration and the shorter use period of the stock suspensions (maximum of one week) resulting in a lower fragmentation and leaching of chemicals. In the present study, both would reduce the load of nanoplastics and chemicals in the experiment, potentially decreasing the toxicity.

A comparison with two other studies investigating the multigenerational effects of plastic particles in daphnids highlights the impact of food limitation. In *D. magna* exposed to 20 nm PS beads (50 mg L\(^{-1} \), ca. 1.1 × 10\(^{13} \) particles mL\(^{-1} \)) in F0 followed by a two-generation recovery, the reproduction but not the growth was negatively affected.\(^{27} \) In *D. pulex*, the exposure to 71 nm PS beads (1 μg L\(^{-1} \), ca. 5 × 10\(^{8} \) particles mL\(^{-1} \)) over three generations induced a higher reproduction and a lower growth in F2 daphnids.\(^{28} \) While these two studies are not directly comparable to ours (beads vs fragments, nanovs microplastics, etc.), we observed much stronger multigenerational effects due to food limitation. This highlights that an environmentally more realistic scenario in which food is scarce might exacerbate the toxicity of plastic particles.

**Toxicity of Aged Microplastics.** The question of whether and how aging affects the MP toxicity is the key to better understand their environmental risks.\(^{1} \) However, only few toxicity studies address this question so far.\(^{29} \) Here, the aging during wastewater treatment is particularly interesting as wastewater is considered a major point source of MPs.\(^{30} \) In previous studies, an incubation of plastic particles with dissolved organic matter (e.g., humic acid, wastewater, and
river water) either reduced their toxicity to microalgae, fairy shrimps, and rotifers or did not induce effects different from pristine MPs in duckweeds and zebrafish. This implies that the aging of plastic particles reduces or does not change their toxicity in a range of species.

These findings are similar for daphnids: after the adsorption of humic substances, nanoplastics and MPs had a lower acute toxicity in *D. magna*. Weathering polyethylene MPs from a facial scrub in a landfill leachate as well as the spring, river, and wastewater did not increase the acute toxicity in daphnids compared to pristine MPs. Recently, Monikh et al. found that dissolved organic matter mitigated the acute toxicity of nanoplastics and silver ions in *D. magna*. Whereas these are all short-term studies, our findings add to this by demonstrating that aged MPs also have a lower effect on the survival in a chronic and multigenerational exposure scenario. While the evidence for a lower toxicity of plastic particles after incubation with dissolved organic matter seems very consistent, this may not be a general pattern. Nasser and Lynch observed a higher mortality when they exposed *D. magna* to functionalized nanoplastics coated with an ecocorona from daphnid biomolecules. The conditioned nanoplastics had a longer gut retention time, which can be the reason for the higher toxicity. This indicates that different types of coronae (dissolved organic matter vs biomolecules) can change the toxicity in very different directions during the aging processes.

Importantly, studies with engineered nanomaterials have already addressed similar questions. Two studies with titanium dioxide and silver nanomaterials recently applied a multigenerational design similar to ours and showed that nanomaterials aged in class V lowland water or in model wastewater treatment plants have a lower overall toxicity in daphnids than pristine nanomaterials. The consistency with our results points toward a common process by which aging reduces the toxicity of plastic and engineered particles alike. If correct, this opens up opportunities for a read-across approach for comparing the hazards of (aged) synthetic particles.

Overall, most available studies on aging of microplastics and nanomaterials are in accordance with our results. Even though the knowledge on the long-term, multigenerational effects of aged particles is still limited, there is a general trend toward an unaltered or lower toxicity to daphnids after aging alone or in the presence of dissolved organic matter.

**Causes for a Lower Toxicity of Wastewater-Incubated Microplastics.** When designing this study, we considered three processes that could alter the toxicity of wastewater-incubated compared to pristine MPs: the sorption and consecutive desorption of wastewater-borne chemicals that would increase toxicity as well as the sorption of dissolved organic matter and biofilm formation that would result in lower effects.

The vector hypothesis states that the sorption of chemicals to MPs and their subsequent desorption after ingestion by an animal increases the exposure to these chemicals and thus, exacerbates the toxicity of MPs. Our results do not support this idea as the MPs incubated for 38 h in wastewater were less toxic than pristine MPs with regard to mortality and similarly toxic regarding the other endpoints. Because wastewater contains hundreds to thousands of so-called micropollutants, we did not investigate the ad-, ab-, or desorption of the specific chemicals. However, previous research has shown that polyacrylic beads efficiently remove the toxicity (as a sum parameter for chemicals) from raw wastewater within 2–6 h. While PS fragments and polymer-specific properties will affect sorption, the sorption of a mixture of wastewater-borne compounds to MPs in our study is very probable. Accordingly, the lack of a higher toxicity of wastewater- incubated MPs can be attributed to a low desorption of chemicals (even though we used a clean medium), a low partitioning of chemicals to daphnids, and/or a low toxicity of the desorbed chemicals in daphnids.

Dissolved organic matter, a complex mixture of high molecular-weight compounds such as humic substances, proteins, and free amino acids, is abundant in aquatic ecosystems. It adsorbs to MPs (e.g., Abdurahman et al.) and can form an ecocorona that is thought to change the toxicity of MPs. Based on previous research with PS MPs and humic acids, we expected that the adsorption of dissolved organic matter from wastewater to MP would result in a shift of their zeta potentials. Contrary to our expectation, the zeta potentials of the pristine and wastewater-incubated PS MPs were similar. Whereas this may imply that the adsorption of dissolved organic matter from wastewater to MP is negligible, a more recent study using fluorescence spectroscopy demonstrated that humic acid readily adsorbs to PS MPs while the zeta potentials remained unchanged. In a follow up study, incubating MPs with various humic substances did not change the zeta potential either. Accordingly, it is reasonable to assume that dissolved organic matter adsorbed to MPs in our study. This is further supported by the observation that an incubation in wastewater improved the stability of the MP suspension.

The third process that can influence the interaction of MPs with the daphnids is biofilm formation during the incubation and/or the experiment. This could affect the toxicity in two ways: the biofilm could provide additional nutrition relieving the stress of food limitation and/or change the surface structure of the MP that reduces the mechanical damage caused by irregular MPs. The SEM micrographs of the MPs taken after the incubation in wastewater (Figure 1) and throughout the use period in M4 medium (Figure S6) show no evidence of biofilm formation, thus, refuting biofilm formation as a relevant factor.

Accordingly, the differences in behavior and toxicity of the pristine and wastewater-incubated MPs were unrelated to the physicochemical properties we analyzed (particle size, surface charge and structure, and biofilm formation). Based on the better dispersion of wastewater-incubated MPs, we believe that the adsorption of dissolved organic matter may be a key factor modulating the toxicity. As this may not affect zeta potentials, other techniques, such as fluorescence spectroscopy, might be better suited for characterizing dissolved organic matter on MPs.

Interestingly, the more stable dispersion of wastewater-incubated MP results in a higher bioavailability of aged MPs and thus, also a higher exposure of daphnids. While we did not quantify the uptake of both MP types, it is worth noting that this resulted in a lower mortality. In addition, it is interesting to find out why the two particle types differed markedly in their effect on the survival but not the other endpoints. That implies two independent modes of action of the MPs of which the one influencing mortality is buffered by the incubation in wastewater, while the one affecting the other endpoints is not.
ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c07911.

Particle size and calculated mass distributions for the microplastics used in this study (Figure S1); zeta potentials of the microplastics used in this study (Figure S2); FTIR spectra of the raw material before and after grinding (Figure S3); FTIR spectra of the microplastics used in this study (Figure S4); photograph of stock suspensions of microplastics suspended in M4 medium that were previously incubated in ultrapure water and wastewater (Figure S5); scanning electron microscopy images of microplastics incubated in ultrapure water and wastewater after suspension in M4 medium for 0, 2, 7, and 9 days (Figure S6); extension of Figure 3 showing the reproduction of D. magna control animals at two different food levels over four generations (Figure S7); timing of the first reproduction of the daphnids exposed to microplastics over four generation (Figure S8); reproductive frequency of the daphnids exposed to microplastics over four generations (Figure S9); body lengths of neonates of the fourth brood of D. magna exposed to microplastics over four generations (Figure S10); growth curves for daphnids in the two growth experiments over 21 days originating from cultures held at either high or low food levels without added particles (Figure S11); data for the endpoints survival, reproduction, and size of an adult D. magna exposed to microplastics over four generations (Table S1); neonate body length and sample sizes of the fourth brood of D. magna exposed to microplastics over four generations (Table S2); and R-code used to produce the predictions underlying the growth curves (PDF).

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Conceptualization, methodology, investigation, formal analysis, visualization, and writing of the original draft including review and editing were done by C.S.; investigation and writing (review and editing) were done by C.W., M.B., J.W., and M.S.; funding acquisition, project administration, supervision, and writing (review and editing) were done by J.O.; and funding acquisition, conceptualization, methodology, writing of original draft, and writing of review and editing were done by M.W.

Notes

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