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What are the drivers of microplastic toxicity? Comparing the toxicity of plastic chemicals and particles to *Daphnia magna*☆

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ABSTRACT

Given the ubiquitous presence of microplastics in aquatic environments, an evaluation of their toxicity is essential. Microplastics are a heterogeneous set of materials that differ not only in particle properties, like size and shape, but also in chemical composition, including polymers, additives and side products. Thus far, it remains unknown whether the plastic chemicals or the particle itself are the driving factor for microplastic toxicity. To address this question, we exposed *Daphnia magna* for 21 days to irregular polyvinyl chloride (PVC), polyurethane (PUR) and polylactic acid (PLA) microplastics as well as to natural kaolin particles in high concentrations (10, 50, 100, 500 mg/L, $\leq$ 59 μm) and different exposure scenarios, including microplastics and microplastics without extractable chemicals as well as the extracted and migrating chemicals alone. All three microplastic types negatively affected the life-history of *D. magna*. However, this toxicity depended on the endpoint and the material. While PVC had the largest effect on reproduction, PLA reduced survival most effectively. The latter indicates that bio-based and biodegradable plastics can be as toxic as their conventional counterparts. The natural particle kaolin was less toxic than microplastics when comparing numerical concentrations. Importantly, the contribution of plastic chemicals to the toxicity was also plastic type-specific. While we can attribute effects of PVC to the chemicals used in the material, effects of PUR and PLA plastics were induced by the mere particle. Our study demonstrates that plastic chemicals can drive microplastic toxicity. This highlights the importance of considering the individual chemical composition of plastics when assessing their environmental risks. Our results suggest that less studied polymer types, like PVC and PUR, as well as bioplastics are of particular toxicological relevance and should get a higher priority in ecotoxicological studies.

1. Introduction

Microplastics are ubiquitous in natural environments and experimental studies have shown that they can induce a wide range of negative impacts in marine and freshwater species across the animal kingdom (Sá et al., 2018; Triebskorn et al., 2019). However, the evaluation of toxicity is complicated by the fact that microplastics are not one homogenous entity (Lambert et al., 2017). They originate from many different product types, are composed of various polymers, chemical additives and side products and differ in particle properties (Rochman et al., 2019). Up to date, few studies have addressed this heterogeneity of materials from a comparative perspective. As an example, the effects of mostly spherical microplastics are investigated. In contrast, irregular fragments and fibers originating from abrasion and fragmentation of plastic products (secondary microplastics) are predominant in the environment but less frequently considered (Burns and Boxall, 2018). At the same time, irregular microplastics might be more toxic than their spherical counterparts, for instance in terms of acute (Frydkjær et al., 2017) and chronic effects in daphnids (Ogonowski et al., 2016). In addition, research focuses only on few polymer types, most often on polystyrene (PS) and polyethylene (PE) particles, disregarding other polymer types of high production and consumption, such as polypropylene (PP) and polyvinyl chloride (PVC; PlasticsEurope, 2015; Sá et al., 2018). However, the toxicity of

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microplastics may also depend on the polymer type or on the chemicals that a plastic product, and therefore its fragments, contain (Renzi et al., 2019). One single plastic product can contain hundreds of chemicals (Zimmermann et al., 2019). These include additives like antioxidants, flame retardants, plasticizers and colorants as well as residual monomers and oligomers, side-products of polymerization and compounding and impurities (Muncke, 2009). Most of them are bound to the polymer matrix only via weak van der Waals forces and, therefore, can leach into the surrounding environment and become available for aquatic organisms (Andrady, 2011; Oehlmann et al., 2009). Once taken up, these plastic chemicals can entail negative impacts. For instance, aqueous leachates from epoxy resin or PVC plastic products induced acute toxicity in *Daphnia magna* (Lithner et al., 2012). Still, studies on the contribution of plastic chemicals to microplastic toxicity are scarce.

Thus, our study aims to elucidate whether the chemicals present in plastics contribute to microplastic toxicity in the well-studied model organism *D. magna*. We produced irregular microplastics from three polymer types that are less frequently studied, including polyurethane (PUR) and polyvinyl chloride (PVC) that often contain high amounts of chemicals (Zimmermann et al., 2019) as well as the bio-based, biodegradable polylactic acid (PLA). We also included kaolin particles as a reference to evaluate whether microplastics are more toxic than natural particles. Since our aim was to compare the contribution of plastic chemicals and particles to the toxicity, we used high concentrations that are not environmentally relevant but induced adverse effects. First, we compared the effects of all microplastic types on mortality, reproductive output, timing of reproduction and body lengths of *D. magna* in a chronic exposure experiment. In a second experiment, we evaluated whether plastics chemicals contribute to microplastic toxicity. For this, we studied the effects of untreated microplastics and microplastics from which we removed the extractable chemicals as well as the extractable chemicals (worst-case scenario) and the chemicals migrating into water (realistic scenario), alone.

2. Materials & methods

2.1. Test materials

We purchased a floor covering, a scouring pad and a shampoo bottle in local retailer stores to produce irregular microplastics. The products are made of petroleum-based PVC and PUR as well as the bio-based and biodegradable PLA. These materials were selected based on our previous results in the Microtox assay (Zimmermann et al., 2019). In the assay the inhibition of bioluminescence of the bacterium *A. fischeri* indicates baseline toxicity. Since the latter generally correlates well with toxicity in *D. magna* (Kaiser, 1998), we chose products that induced a high baseline toxicity in the Microtox assay (Zimmermann et al., 2019, PVC corresponds to PVC 4, PUR to PUR 1, PLA to PLA 3). In our previous study, we confirmed the polymer types using Fourier-transform infrared spectroscopy and characterized the chemicals present in the products by performing non-target, high-resolution gas chromatography–mass spectrometry.

2.2. Production of microplastics

Whenever feasible, we used glass consumables to avoid sample contamination, rinsed all materials twice with acetone (pico-grade, LGC Standards) and annealed glass items at 200 °C for ≥3 h. The content was removed from packaging samples and the products were rinsed thoroughly with ultrapure water until all residues were removed. Plastic items were cut into small pieces (~0.5 cm^2), frozen in liquid nitrogen and ground in a ball mill (Retsch MM400, Retsch GmbH, Germany) at 30 Hz for 1 min. The process of freezing and grinding was repeated 6–10 times to produce sufficient amounts of plastic powder. The plastic powder and kaolin (~$Al_2Si_2O_5(OH)_4$, CAS 1332-58-7, Merck, Darmstadt, Germany) were sieved to ≤59 μm for particle characterization and the experiments. To this end, polyester mesh (RCT Reichelt Chemie Technik GmbH + Co, Heidelberg, Germany) with respective mesh sizes were fixed horizontally in a custom-made sieving device that was mounted on a sediment shaker (Retsch AS 200 basic, Retsch GmbH, Germany) and was shaken at 80–100 Hz for 10 min. With a size of ≤59 μm all particles are in a size range which can be ingested by *D. magna* (Burns, 1968).

2.3. Preparation and characterization of stock suspensions

We prepared microplastic stocks by suspending between 0.2 and 500 mg of particles/L Elendt M4 medium (Elendt and Bias, 1990) and shaking it at 80 rpm for ≥24 h (GFL-Kreis-Schüttler 3017, Gesellschaft für Labortechnik GmbH, Burgwedel, Germany). We used mass-based concentrations, because we aimed at comparing the toxicity of the chemicals present in the different plastics based on the same mass, not particle number. The corresponding numerical particle concentrations and size distributions were also determined using a Coulter counter (Multisizer 3, Beckman Coulter, Germany; orifice tube with 100 and/or 400 aperture diameter for a particle size range of 2.0–60 μm and 8.0–240 μm, respectively). For this, 1.0–2.5 mL of the particle suspension were taken from the middle of the exposure vessel or flask (continuously stirred) and transferred immediately to the Coulter counter medium (100 mL sterile-filtrated 0.98% sodium chloride, continuously stirred). In addition to the samples, we also analyzed the pure sodium chloride as a blank and the Elendt M4 medium as microplastic-free control medium. The kaolin particles were treated identically like the microplastics. All samples were analyzed in three to ten replicates. The blank corresponding to each measurement was analyzed in triplicates.

2.4. Microplastic characterization

For an initial characterization and comparison of our microplastics regarding size distribution, shape, surface morphology and behavior in suspension, we prepared suspensions with 0.2, 2.0, 20.0, 60.0 (not measured for PLA and kaolin), 100 and 500 mg microplastics or kaolin/L Elendt M4 medium. We determined particle size distributions (Fig. S1) as well as numerical particle concentrations using a Coulter counter (see 2.3.). From the latter, we obtained calibration curves by linear regression for mass (mg) vs. numerical particle concentration/L for each plastic type. We corrected the latter for the mean particle concentration in the respective control measurement (microplastic-free Elendt M4 medium; Fig. S2). In order to assess particle shape and surface morphology, we took images with a Hitachi S-4500 scanning electron microscope (SEM; Fig. 1). Additionally, stock suspension containing 500 mg microplastics or kaolin/L were visually examined for the distribution of particles in the water column and for agglomeration immediately after shaking and after resting for two and seven days.

2.5. Culture of test organism Daphnia magna

D. magna were obtained from IBACON GmbH (Rossdorf, Germany). Ten individuals were cultured in 1 L of Elendt M4 medium (Elendt and Bias, 1990) at a constant temperature of 20 ± 1 °C and a photo-period of 16:8 h light:dark for approximately 28 days. Juveniles were removed thrice a week and daphnids were fed with a suspension of live green algae (*Desmodesmus subspicatus*), cultured

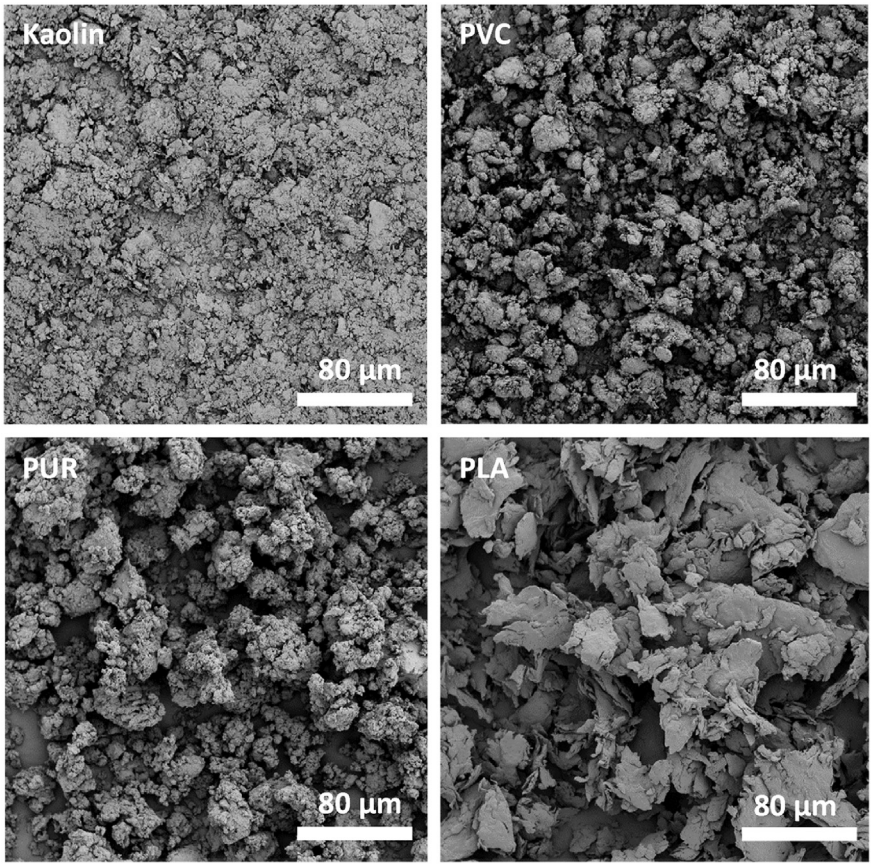

Fig. 1. Scanning electron microscope (SEM) images of kaolin as well as PVC, PUR and PLA microplastics (300 × magnification).

according to OECD guideline (OECD, 2012) supplying 0.15 mg carbon per individual per day. Once a week, the medium was completely renewed.

2.6. Chronic toxicity of microplastics on Daphnia magna

Prior to toxicity experiments, we evaluated qualitatively whether *D. magna* ingests PVC, PUR and PLA microplastics. PVC and PLA particles were stained with Nile red (CAS 7385-67-3, reinst; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for visualization adapting the method of Erni-Cassola et al. (2017). Six starved individuals which were 6 d old were exposed to a 250 mg/L microplastic suspension at culturing conditions. After 24 h, we analyzed ingestion using an Olympus BX50 fluorescence microscope (Olympus Europa SE & Co. KG, Hamburg, Germany).

To analyze and compare the effects of microplastics and kaolin particles, we conducted chronic exposure experiments with *D. magna* according to OECD guideline 211 (OECD, 2012). In brief, neonates (<24 h old) of the third or fourth brood were exposed individually for 21 d in 100 mL glass beakers containing 50 mL Elendt M4 medium. Microplastic suspensions were prepared as stocks and continuously stirred during the transfer to the test vessels. After dilution with Elendt M4 medium to the desired exposure concentrations of 10, 50, 100 and 500 mg/L, we determined the size distributions (see 2.3) and the numerical particle concentrations corrected for the mean particle concentration in the control (Elendt M4 medium, Table S1). We selected such high concentrations because they induced adverse effects in *D. magna* in previous experiments conducted in our laboratory (unpublished data). We used 10 replicates per treatment and 20 negative controls (NC) in each experiment. Experiments were conducted at a 16:8 h light:dark cycle at 20 ± 1 °C and beakers were covered with watch glasses to reduce evaporation. Animals were fed daily with *D. subspicatus* according to OECD guideline 211 (OECD, 2012) and the test medium was completely renewed thrice a week by transferring the daphnids into new vessels. Each day, we recorded the mortality of adult daphnids (15 s immobility after agitation; OECD, 2004) and their reproductive output (number of neonates per female). We also recorded the day of first brood (timing of reproduction) and the total number of live offspring for each surviving parent organisms throughout the experiment. 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). We observed that eight out of 180 individuals, randomly distributed across all treatments, had >40% lower body length compared to the other animals and did not reproduce. We sexed these animals according to Mitchell (2001) and identified them as females. Microplastic concentrations reducing the reproduction by 50% compared to the negative control ($EC_{50Repro}$) were used in the second experiment (2.7.). We excluded the smaller individuals mentioned above from the calculation of the $EC_{50Repro}$ because we could not estimate an EC_{50} when they were included.

2.7. Contribution of plastic chemicals to microplastic toxicity

In order to analyze whether the chemicals present in and leaching from plastics induce the observed effects, we conducted a second chronic exposure experiment with *D. magna*. Generally, the setup and endpoints were identical as before (2.6.) but in this second experiment daphnids were exposed to four treatments reflecting four exposure scenarios (Fig. 2):

(1) PVC, PUR and PLA microplastics containing all chemicals (MP).
(2) PVC, PUR and PLA microplastics extracted with methanol. Thus, they do not contain extractable chemicals (eMP).
(3) The corresponding plastic extracts (E) containing all chemicals that can be extracted with methanol. The extracts represent a worst-case scenario because extraction with an organic solvent will release most of the chemicals present in the material.
(4) Plastic migrates (M) containing the chemicals released from PVC, PUR and PLA microplastics into the water, thus, representing a more realistic scenario.

For preparing the suspensions (MP, eMP) and leachates (E, M) of each microplastic type, we used the respective mass concentrations

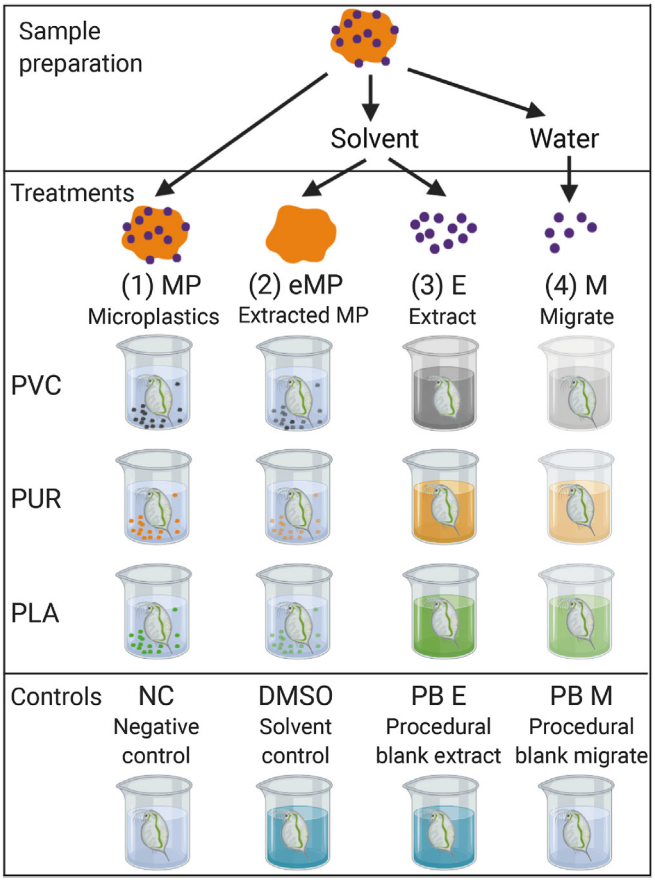

Fig. 2. Setup of the second experiment. Daphnids were exposed to four treatments of PVC, PUR and PLA: (1, MP) untreated microplastics containing all chemicals, (2, eMP) microplastics without extractable chemicals, (3, E) plastic extracts containing all extractable chemicals and (4, M) plastic migrates containing the chemicals released from microplastics into water (M). We included a negative control (NC), a solvent control (DMSO) and procedural blanks of the extraction (PB E) and migration (PB M) consisting of Elendt M4 media treated identically as the plastic extracts and migrates, respectively.

that reduced reproduction by half in the first experiment ($EC_{50Repro}$, PVC: 45.5 mg/L, PUR: 236 mg/L, PLA: 122 mg/L). This means that for each microplastic type, suspensions for scenario 1 and 2 were prepared using the same mass concentrations. Scenarios 3 and 4 contained the chemicals extracted or migrating from the very same mass to ensure comparability. Specifically, the suspensions and leachates for the four exposure scenarios were prepared as follows:

(1) MP stock suspensions were prepared as described in 2.3.

(2 + 3) Extracted microplastics and the extracts were produced by weighing microplastics in amber glass vials and adding 13.5 mL methanol (99.9% LC-grade, Sigma-Aldrich, exception PUR: 16.5 mL). We selected methanol as solvent because it does not dissolve the polymers. After sonication in an ultrasound bath for 1 h at room temperature, the suspensions were vacuum-filtrated over a polyethersulfone membrane (pore size: 1 μm, Sartorius Biolab Products, Satorius Stedim Biotech GmbH, Goettingen, Germany) pre-calibrated with methanol to separate the extract from the extracted particles. The extracted particles were dried at 30 °C for 24 h, the dry weight was recorded and eMP stock suspensions were prepared as described in 2.3. The extracts were transferred into clean glass vials and dimethyl sulfoxide (DMSO, Uvasol, Merck) was added as a keeper. The volume of DMSO was dependent on the recovered extract volume to adjust to the plastic concentrations corresponding to the $EC_{50Repro}$ used in scenarios 1 and 2. Extracts were evaporated under a gentle stream of nitrogen and stored at −20 °C prior to use. Exposure vessels were spiked with 5 μl extract.

(4) Migrates were prepared by suspending microplastic masses corresponding to the $EC_{50Repro}$ used in scenarios 1 and 2 in 5.5 L Elendt M4 medium 48 h before the start of the experiment. Directly prior to the initial set up of the experiment as well as each medium renewal, 500 mL of that migrate suspensions were filtrated over a polyethersulfone membrane with a pore size of 1 μm to remove the particles and 50 mL aqueous migrate were transferred into each test vessel. In that way, the migration of chemicals proceeded in parallel to the experiment.

In order to exclude effects of the solvent or a potential contamination, we included a solvent control (DMSO only) and procedural blanks of the extraction (PB E) and the migration (PB M) consisting of Elendt M4 media treated identically as the plastic extracts and migrates, respectively.

2.8. Data analysis

We used GraphPad Prism 5 (GraphPad Software, San Diego, CA) for regressions and statistical analyses. Continuous life-history data were checked for normal distribution (D'Agostino-Pearson tests for $n \geq 8$ or Kolmogorov-Smirnov tests for $n = 5–7$). Since all data was not normally distributed, we used non-parametric Kruskal-Wallis with Dunn's multiple comparison post-test to assess differences between treatments and negative controls. Fisher's exact test was applied for categorical data. The significance level was set at $p < 0.05$. The 10% and 50% effect concentrations (EC_{10} and EC_{50}) for reproduction were determined using a four-parameter logistic model and were compared using the extra sum-of-squares F test. We indicate the F value together with the degrees of freedom numerator (DFn) and denominator (DFd). Since solvent control (DMSO), extraction (PB E) and migration (PB M) procedural blanks did not differ significantly from the negative control, we pooled all controls (C).

3. Results

3.1. Characterization of microplastics

To characterize the microplastics and kaolin used in our study, we compared the numerical particle concentrations at identical mass concentrations, the size distributions, shapes and surface morphology as well as behavior in suspension prior to experiments. For the highest mass-based concentration (500 mg/L), the numerical concentrations were 8.38×10^7 particles/L (PUR), 1.35×10^8 particles/L (PVC) and 2.08×10^8 particles/L (PLA, Fig. S2). Thus, the PLA suspension contained 1.6 times more particles than the PVC suspension and 2.5 times more than the PUR suspension. While at 100 mg/L, the numerical concentrations of all microplastics were very similar and only differed by a maximum factor of 1.2, the differences increased again towards lower mass concentrations. Correspondingly, at the lowest mass-based concentration (0.2 mg/L), numerical concentration were 3.77×10^6 particles/L (PLA), 1.35×10^7 particles/L (PUR) and 1.63×10^7 particles/L (PVC). That 100 mg/L concentrations were most similar to each other while differences between microplastic types increased towards lower and higher concentrations was also true for the concentrations in the exposure vessels. Here, the numerical concentrations varied by a maximum factor of 4.0 for 10 mg/L, of 1.8 for 100 mg/L and 2.2 for 500 mg/L between the three polymers (Table S1). In contrast, kaolin suspensions contained 11–50 times more particles at same mass concentrations.

The size distributions of all microplastics of our study are very similar (Fig. S1). Independent of the particle type, the number of particles increases with decreasing sizes. Whereas the majority of kaolin particles is <10 μm, microplastics contain higher relative particle quantities at sizes up to about 20 (PVC) or 40 μm (PUR, PLA). All particles have irregular shapes and rough surfaces (Fig. 1). While PVC, PUR and kaolin particles are rather round, PLA particles are flatter and disc-shaped. After preparation of stocks, including ≥24 h of shaking, all microplastic types and kaolin were homogenously distributed in the water column. Kaolin remained suspended in the water phase after two and seven days without moving the suspensions, whereas most microplastics sedimented and few floated on the surface. Although daphnids are primarily filter feeders, they also graze on sediments and we observed them at the bottom of the test vessels. Thus, all microplastic types are available to the daphnids. A qualitative uptake experiment demonstrated that PVC, PUR and PLA microplastics are readily ingested by *D. magna* since they were visible in the gastrointestinal tract (Fig. S3).

3.2. Chronic effects of microplastics on Daphnia magna

To investigate whether microplastics affect life-history traits of *D. magna* and whether toxicity changes with the plastic type, we exposed daphnids to PVC, PUR, PLA and kaolin particles. All microplastics reduced the reproductive output of *D. magna* (Fig. 3A) with an efficiency and effect level specific to the plastic type. PVC impaired the reproduction the most with an EC_{50} of 45.5 mg/L (Table 1) and significantly decreased the number of neonates from 101 per adult (control) to 34 at 100 mg/L and to 0 at 500 mg/L (Fig. 3A). Exposure to PLA and PUR microplastics reduced the reproduction significantly compared to the control at 500 mg/L with EC_{50} values of 122 and 236 mg/L, respectively. While an exposure to 10 and 50 mg/L of kaolin increased the reproduction to 130 and 110 neonates/animal ($p > 0.05$), 500 mg/L significantly reduced the mean number of neonates per surviving female (21 neonates/animal) to values similar to PLA. With an EC_{50} of 275 mg/L, kaolin was less efficient than microplastics in affecting reproduction. In addition, exposure to 500 mg/L PVC and kaolin significantly delayed the reproduction and the mean day of the first brood occurred eight and four days later than in the control animals, respectively (Fig. S4).

Using the same data, we also compared the reproductive output based on numerical particle concentrations (Fig. 3B). With an EC_{50} of 1.14×10^7 particles/L, PVC was most efficient in reducing the reproduction, followed by PLA (EC_{50} of 5.13×10^7 particles/L) and PUR (EC_{50} of 7.29×10^7 particles/L, Table 1). With an EC_{50} of 2.61×10^9 particles/L, kaolin was >35 times less toxic than all three microplastics. A statistical comparison of the EC_{50} values of the four particle types demonstrated that all values, both, if based on masses ($F = 9.09$ ($DFn = 3$, $DFd = 119$)) or numerical particle concentration ($F = 61.76$ ($DFn = 4$, $DFd = 135$)), differed significantly from each other ($p < 0.05$).

Except for PLA, the impacts of the particle exposure on daphnid survival were low with 10 mg/L PVC and 50 mg/L kaolin inducing a maximum of 30% mortality (Fig. S5). An exposure to PLA increased the mortality in a concentration-dependent manner to 60% at 500 mg/L. The mortality in the controls was 5%.

The mean body length of adult *D. magna* was significantly lower in animals exposed to 500 mg/L of microplastics (Fig. S6). Control animals were 4.10 mm in size compared to 3.48, 3.57 and 3.30 mm in specimens exposed to PVC, PUR and PLA, respectively. Exposure to the 500 mg kaolin/L also reduced the size of daphnids similar to PLA.

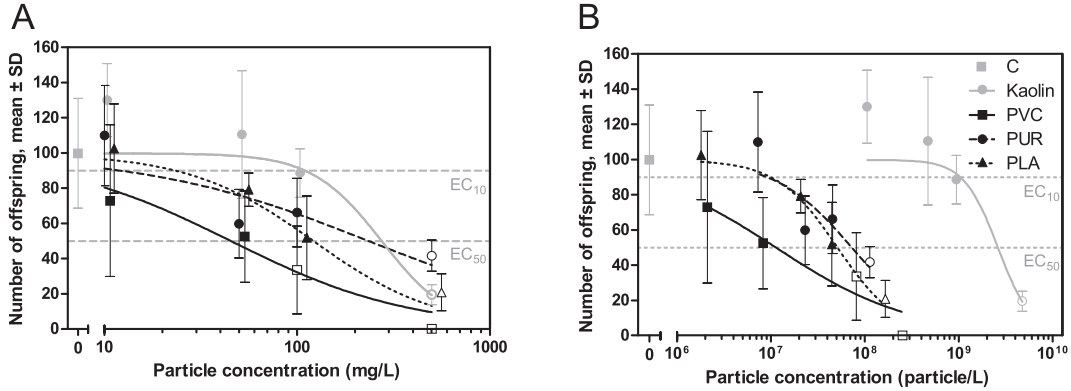

Fig. 3. Effects of a chronic exposure of *Daphnia magna* to kaolin, PVC, PUR and PLA particles on the reproduction. Data is presented as mass-based (A) and numerical concentrations (B). The latter were corrected for mean particle concentration in the blank (M4 medium). Open symbols indicate significant differences ($p < 0.01$) compared to control animals (C). EC_{10}, EC_{50}: concentrations inducing 10 and 50% effect, SD: standard deviation.

Table 1
Mass-based and numerical particle concentrations of kaolin as well as PVC, PUR and PLA microplastics reducing the reproduction of *Daphnia magna* by 10% (EC_{10}) and 50% (EC_{50}).

Treatment	EC_{10} (mg/L)	EC_{50} (mg/L)	EC_{10} (particle/L)	EC_{50} (particle/L)
Kaolin	111 (43.8–279)	275 (174–435)	1.05×10^9 (4.12×10^8–2.66×10^9)	2.61×10^9 (1.64×10^9–4.14×10^9)
PVC	n.a.[a]	45.5 (26.8–77.2)	n.a.[a]	1.14×10^7 (6.96×10^6–2.61×10^7)
PUR	12.4 (3.01–50.7)	236 (120–463)	9.26×10^6 (3.26×10^6–2.63×10^7)	7.29×10^7 (4.58×10^7–1.16×10^8)
PLA	23.6 (9.62–58.0)	122 (79.9–186)	1.07×10^7 (3.97×10^6–2.56×10^7)	5.13×10^7 (3.50×10^7–7.50×10^7)

[a] EC_{10} below the lowest measured concentration of 10 mg/L; The 95% confidence intervals are given in brackets.

3.3. Contribution of plastic chemicals to microplastic toxicity

Next, we evaluated whether the observed toxicities of microplastics are caused by plastic chemicals. For this, we exposed *D. magna* to microplastics containing all chemicals (MP), extracted microplastic particles (eMP), the chemicals extracted from PVC, PUR and PLA microplastics (E) and the chemicals migrating from the microplastics to aqueous medium (M, Fig. 2). In order to ensure comparability, the exposure concentrations were based on the $EC_{50Repro}$ that we derived from the first experiment (PVC: 45.5 mg/L; PUR: 236 mg/L, PLA: 122 mg/L).

For PVC, exposure to the extracted chemicals (E) but not the plastic particles (MP and eMP) reduced significantly the reproductive output from 117 (control) to 25 neonates/animal (Fig. 4A). Along that line, exposure to the PVC extract (E) also delayed the reproduction by three days (Fig. 4D) and reduced the body lengths of daphnids (4.08 vs. 4.56 mm in control animals, Fig. S7A). The chemicals migrating to aqueous medium (M) did not have a significant effect.

In comparison, the toxicity of PUR and PLA microplastics in *D. magna* was mediated by the particle properties and not the chemicals. Here, the microplastics and extracted microplastics significantly reduced the reproduction (Fig. 4B and C) as well as the size of daphnids (Figs. S7B and C). Extracted PUR particles also delayed the day of the first brood by 1–3 days compared to the control (Fig. 4E). In line with the first experiment, PLA was the only microplastic type inducing mortality. This effect was mediated by the particles and not the chemicals (Fig. 5).

To further evaluate if and which particle characteristics might be responsible for the deviating toxicities, we analyzed differences in numerical concentrations (particle count), size distribution as well as the shape and surface morphology of original and extracted microplastics. Regarding particle numbers, the suspension of extracted PVC particles contained 1.89×10^8 particles/L compared to 0.50×10^8 particles/L in the suspension of the PVC microplastics (Fig. 6). Although both suspensions were prepared using the same mass, the extracted microplastic suspension had a 3.8 times higher particle concentration. Comparisons of the particle size

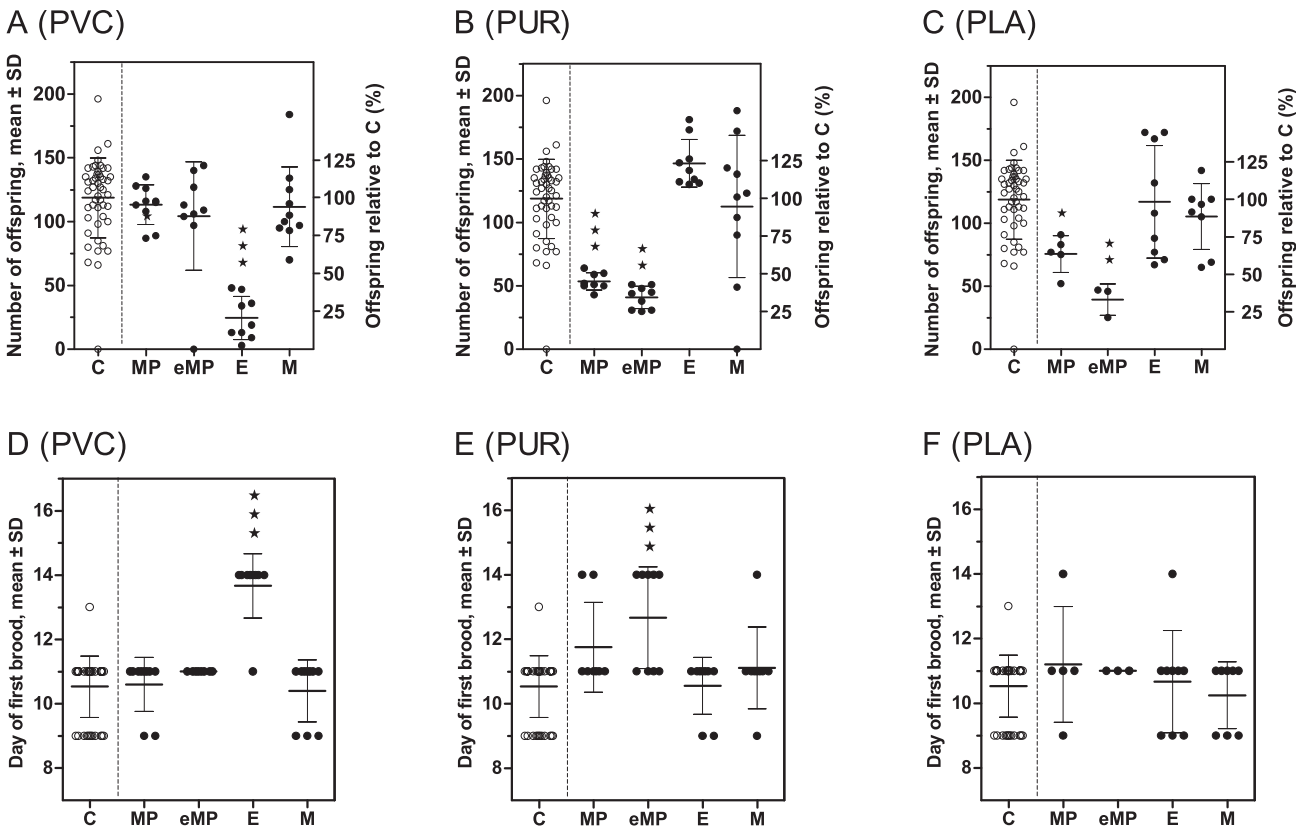

Fig. 4. Effect of a chronic exposure of *Daphnia magna* to PVC (45.5 mg/L), PUR (236 mg/L) and PLA (122 mg/L) microplastics on the reproductive output (A–C) and the timing of reproduction (D–F). Treatments include microplastics (MP), microplastics without extractable chemicals (eMP), the chemicals extracted (E) and migrating from microplastics to aqueous medium (M). Asterisks indicate significant differences to the controls (C) with ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$ (Kruskal-Wallis with Dunn's multiple comparison post-test).

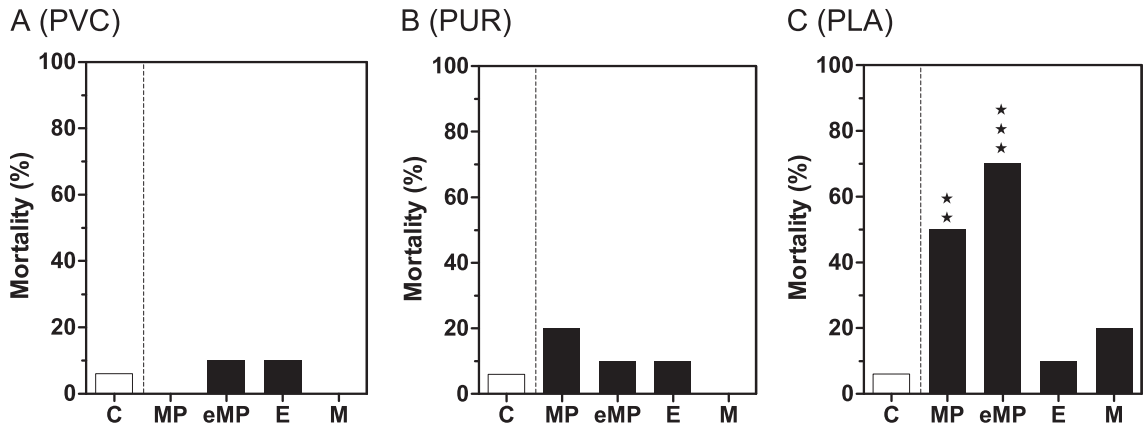

Fig. 5. Mortality of *Daphnia magna* after 21 days exposure to 45.5 mg/L PVC (A), 236 mg/L PUR (B) and 122 mg/L PLA (C) microplastics. Treatments include microplastics (MP), microplastics without extractable chemicals (eMP), the chemicals extracted (E) and migrating from microplastics to aqueous medium (M). Asterisks indicate significant differences to the controls (C): ★★ $p < 0.01$, ★★★ $p < 0.001$ (Fisher's exact test, comparison to C).

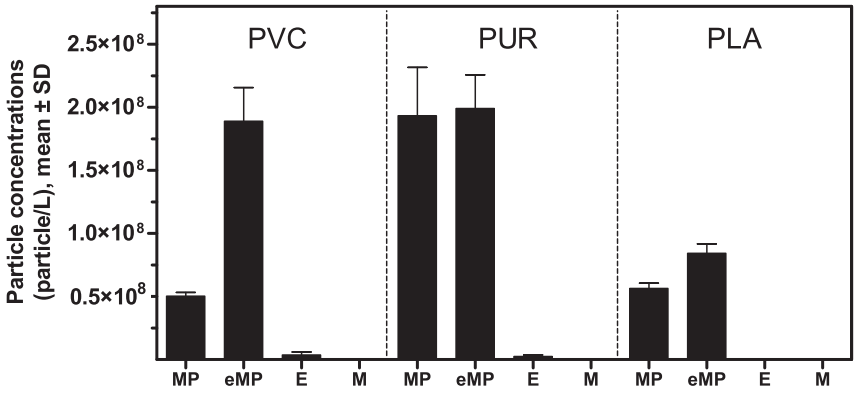

Fig. 6. Numerical particle concentrations in the treatment suspension of the second experiment. Treatments include microplastics (MP), microplastics without extractable chemicals (eMP), the chemicals extracted (E) and migrating from microplastics to aqueous medium (M). SD: standard deviation.

distributions showed that the extracted PVC particles are smaller than the untreated ones (Fig. S8). Suspensions of the original and extracted PUR microplastics contained approximately the same particle concentration (1.93×10^8 and 1.99×10^8 particles/L) like extracted PVC. The numerical concentrations of microplastics in the PLA suspensions were approximately 2.8 times lower (MP: 0.57×10^8 particles/L, eMP: 0.84×10^8 particles/L; Fig. 6). The extraction of PUR and PLA microplastics did not change their size distribution (Fig. S8). SEM imaging demonstrated that the extraction did not alter shapes nor surface morphologies of any of the microplastic types (Fig. S9).

4. Discussion

4.1. Microplastic effects on Daphnia magna depend on the plastic type

For the hazard assessment of microplastics, it is crucial to consider the diverse picture of synthetic polymers entering the environment (Lambert et al., 2017; Rochman et al., 2019). However, the physical and chemical heterogeneity of microplastics has rarely been reflected in ecotoxicological studies to date. To address this knowledge gap, we compared the impact of so far understudied PVC, PUR and PLA particles on *D. magna* upon chronic exposure. Since we aimed at understanding the chemical and physical toxicity of microplastics and not their environmental risks specifically, we used high concentrations that caused negative impacts in *D. magna* but are clearly much higher than currently occurring in freshwater ecosystems.

In this range, all three microplastics affected life-history traits of *D. magna*. While PVC microplastics were most potent in decreasing (at 10–500 mg/L) and delaying reproduction (at 500 mg/L), PLA was in reducing survival (at 500 mg/L). When comparing reproductive outputs based on numerical concentrations, we observed a similar picture, with PVC being more potent in decreasing reproduction ($EC_{50} = 1.14 \times 10^7$ particle/L) than PLA (5.13×10^7 particle/L) and PUR (7.29×10^7 particle/L). Thus, impacts of microplastics depend on the polymer type and the endpoint under investigation.

Besides our toxicity study, only few others have analyzed polymers other than PS and PE or compared different microplastic types. Two studies compared PE and polyethylene terephthalate (PET) microplastics from consumer plastics and observed neither acute effects at mass concentrations comparable to our study (particle size: 23–264 μm; concentration: 100 mg/L; Kokalj et al., 2018) nor chronic impacts (exposure concentration based on surface area; Trotter et al., 2019) on daphnids. So far, toxicity data for PUR particles are unavailable but some data for PVC and PLA microplastics exists. Irregular PLA microplastics (3.4 μm; 19.6 μg/L) did not affect feeding, size and population growth of *D. magna* upon chronic exposure (Gerdes, 2018). In a comparative analysis of irregular PVC, PP and PE particles (10–100 μm; 50 mg/L), PP and PE induced a higher acute toxicity than PVC on *D. magna* under fasting

conditions (Renzi et al., 2019). Schrank et al. (2019) compared irregular rigid and flexible PVC (4–276 μm) and reported delay of primiparity in *D. magna* upon exposure to rigid PVC and alterations in body lengths and reproductive output for flexible PVC. This indicates that the toxicity of microplastics not only depends on the polymers type but also differs between plastics made of the same polymer.

Comparison of microplastics to the natural kaolin particle demonstrates that kaolin particles are less toxic than microplastics. In general, at the same mass concentrations, the numerical concentrations of kaolin were much higher than those of all microplastics in our study. Kaolin impaired reproduction, *Daphnia* size and the day of first brood at much higher particle concentrations (4.75×10^9 particles/L) compared to microplastics. In line with our results, upon acute as well chronic exposure of *D. magna*, irregular microplastics had, respectively, a significant lower LC_{50} (PET; 5 μm; Gerdes et al., 2019) as well as $EC_{50\ Repro}$ (2.6 μm; Ogonowski et al., 2016) value than kaolin. This suggests (1) that the natural kaolin particle is less toxic than microplastics in daphnids and, (2) that the effect is independent of the mere number of particles.

Taken together, other factors than polymer type and numerical particle concentrations, that are specific to each plastic particle, influence adverse effects of microplastics. These may include physical characteristics, such as size, shape and surface morphology, and chemical characteristics, such as the presence of additives and side products.

4.2. Role of chemicals in microplastic toxicity

We aimed at elucidating whether plastic chemicals present in and leaching from the microplastics contribute to their toxicity. For that purpose, we compared within one microplastic type the chronic toxicity of the microplastics to that of particles without extractable chemicals, the chemicals extracted from the microplastics reflecting the chemicals that are used in plastic and can potentially be released in the environment under worst-case conditions. Additionally, we tested the chemicals migrating from plastic in aqueous medium within 21 days reflecting those realistically entering freshwater ecosystems.

Our results demonstrate that chemicals can be the main driver of microplastic toxicity. However, their contribution depends on the plastic type. For the PVC we analyzed, the extractable chemicals caused toxicity since only the plastic extract adversely affected *D. magna*. There was no toxicity when the chemicals were incorporated in the microplastics nor did the chemical toxicity migrate into aqueous medium over a 21-day period. This indicates that under more realistic conditions, the toxicity of leaching chemicals might be limited. However, the quantities and effects of chemicals leaching from plastic debris in natural environments are highly context dependent (e.g., type and surface area of debris, temperature, microbial activity) and difficult to generalize. In addition, it remains to be seen how the effects of chemicals leaching from artificially ground microplastics will translate to plastics aged in nature.

In contrast to PVC, the toxicity induced by the analyzed PUR and PLA was not caused by plastic chemicals since neither the extracted nor migrating compounds had negative impacts. Instead, the microplastics and extracted microplastics induced adverse effects implying that the particle characteristics of PUR and PLA microplastics are causative.

Few studies have compared the physical and chemical toxicity of microplastics. For instance, the negative impacts of PET fibers on survival of *D. magna* (Jemec et al., 2016) and PS beads on reproduction of *C. elegans* (Mueller et al., 2020) were not caused by their chemical leachates. In contrast, Oliviero et al. (2019) linked the toxicity of irregular PVC microplastics made from toys (<20 μm) on sea urchin to the leachable chemicals. Chemical-driven effects were also observed in plants. Here, leachates of polycarbonate (PC) granules but not whole microplastics affected germination of a garden cress (Pflugmacher et al., 2020). In contrast to our PUR particles that do not contain compounds toxic to *D. magna*, other PUR consumer products leached chemicals with acute toxicity to daphnids (Lithner et al., 2009). These studies strengthen the argument that chemicals can drive microplastic toxicity and clarify that the chemical toxicity is specific to the individual material and not necessarily to a polymer type. Nonetheless, there is some evidence that the toxicity of microplastics made of certain polymers, especially PVC and PC, is caused by the plastic chemicals.

In order to find out why only the plastic chemicals in PVC induced toxicity, we compared the chemical profiles of the three plastics (details in Zimmermann et al., 2019). Interestingly, the total abundance (peak area) was largest for the PLA extract followed by PVC and PUR extracts. Likewise, PLA contained 103 compounds, followed by PVC (52) and PUR (44). Thus, neither the abundance nor the number of plastic chemicals predicts the *in vivo* toxicity of plastic extracts observed in this study. We further prioritized the identified chemicals based on their abundance and *in vitro* toxicity and detected high priority chemicals in all three plastics, for instance the plasticizer tributyl acetylcitrate in PVC, the antioxidant butylated hydroxytoluene in PUR and the side product 9-octodecamide in PLA (Zimmermann et al., 2019). However, it still remains elusive whether the toxic effects of PVC on *D. magna* were caused by individual compounds or a mixture of chemicals. Overall, the chemicals inducing *in vivo* effects, likewise as the chemicals inducing *in vitro* toxicity, remain to be identified which makes further research necessary.

4.3. Role of physical characteristics in microplastic toxicity

The physical properties of microplastics, including size, shape, surface morphology and charge, may also play an important role in their toxicity. For instance, 100 nm PS beads were more toxic in *D. magna* than 2 μm PS beads (Rist et al., 2017) and PET fibers induced stronger effects than PE beads in *Ceriodaphnia dubia* (Ziajahromi et al., 2017). Regarding the surface charge, positively-charged amidine 200 nm PS nanobeads were more toxic than negatively charged carboxylated PS beads in *D. magna* (Saavedra et al., 2019). While identifying which physical property drives the toxicity of microplastics is not an easy task, this highlights that multiple factors need to be considered.

In terms of particle size, smaller microplastics did not induce a higher toxicity in our study: The adverse effects of PLA and PUR were induced by particles mostly smaller than 40 μm (MP and eMP) while the smaller PVC particles (mostly <20 μm) did not cause an effect. Compared to the suspension based on PVC microplastics, the one of extracted PVC contained much more small particles, probably as a consequence of fragmentation during extraction, but still was not toxic to *D. magna*. Due to technical limitations, we could not determine the occurrence of particles <2 μm. Thus, the contribution of smaller microplastics and nanoplastics potentially present in the suspensions and extracts remains unknown.

In terms of shape and surface morphology, we generated irregular microplastics from plastic consumer products. Since materials have different fragmentation pattern, creating identical particle shapes is not entirely feasible. Nevertheless, all selected microplastics share an irregular shape and rough surface. Here, PVC and PUR microplastics have a very similar, rounded shape but do not resemble each other with regards to their toxicity. *Vice versa*, PUR and PLA microplastics have a somewhat dissimilar shape but induced a comparable toxicity. Thus, shape is not the driving factor

for toxicity in our study. However, this may be different when investigating particles with more dissimilar shapes (e.g., beads vs. fibers).

Additionally, a higher numerical concentration at equal mass concentrations was not responsible for higher effects. For instance, PLA MP and eMP suspensions had lower numerical concentration than the PVC eMP suspension but PLA and not PVC particles affected life-history traits of *D. magna*. Thus, other particle-related differences of PLA compared to PVC microplastics, like the flatter and more angular shape or another surface charge of PLA, may render them more toxic. In general, the combination of the several physical characteristics specific to each particle type influences microplastic toxicity. This indicates the necessity to consider multiple physical properties of microplastics in future toxicity studies.

Summing up, for the microplastics we studied, the effects of PVC are driven by chemical toxicity while physical toxicity dominates for PUR and PLA microplastics. Concerning the latter, neither a higher numerical concentration, the specific particle size, shape nor surface morphology appears to be the sole relevant factor. Since PVC microplastics were still more toxic than PUR and PLA particles, chemicals seem to have a higher impact than physical properties on microplastic toxicity in our study.

4.4. Bioplastics are not necessarily safer than conventional plastics

Bioplastics are made from renewable resources (bio-based) and/or degrade in the natural environment by the action of microorganisms (biodegradable; Lambert and Wagner, 2017). They are especially prone to end up in natural ecosystems due to the promise that they easily degrade in nature which is often not even true (Haider et al., 2019). Although marketed as a more sustainable alternative, there are first indications from *in vitro* testing that they are not necessarily toxicologically safer than their petroleum-based counterparts (Zimmermann et al., 2019). Our *in vivo* results support that idea as PLA was more toxic than PVC and PUR with regards to daphnid mortality. Besides *D. magna*, also other aquatic organisms are susceptible to PLA microplastics. Exposure of the oyster *Ostrea edulis* to 0.8 or 80 μg/L of 65.6 mm (Green, 2016) and the lugworm *Arenicola marina* of 1.4–707 μm (Green et al., 2016) PLA microplastics resulted in elevated respiratory rates. While we cannot attribute the toxicity of the PLA to plastic chemicals in our study, PLA leachates induced *in vitro* baseline toxicity (Ramot et al., 2016; Zimmermann et al., 2019). This phenomenon is not limited to PLA but also applies to other bioplastics. For instance, aqueous leachates of polybutylene adipate terephthalate (PBAT) and polyhydroxybutyrate (PHB) granules increased the immobility of *D. magna* after 48 h of exposure (Göttermann et al., 2015). Taken together, bioplastics, like PLA, can be similarly toxic as conventional plastics and are especially prone to end up in the environment and therefore, might pose a particular hazard for aquatic organisms.

5. Conclusions

The aim of this study was to characterize the toxicity of microplastics from currently understudied materials as well as to elucidate whether the toxicity is driven by the chemicals present in microplastics. We, thus, chronically exposed *D. magna* to high concentrations of PVC, PUR and PLA microplastics or kaolin as well as to four exposure scenarios to differentiate between physical and chemical toxicity. The latter included untreated microplastics, microplastic particles without extractable chemicals as well as the compounds extracted or migrating from the plastics. All three microplastic types adversely affected the life history of *D. magna* at high concentrations. Here, the magnitudes of effect on multiple endpoints were material-specific with PVC being most toxic to reproduction and PLA inducing most mortality. We demonstrate that plastic chemicals are the main driver for toxicity in case of the PVC but not of the PUR and PLA microplastics investigated here. Additionally, the high mortality upon PLA exposure indicates that bioplastics can be similarly toxic as their conventional counterparts. Our findings highlight that microplastics cannot be treated as homogenous entity when assessing their environmental hazards. Instead, multiple plastic types as well as chemical compositions and physical characteristics of microplastics need to be taken into account. Importantly, studying the toxicity of other polymers than PS and PE, especially bioplastics, is particularly relevant.

CRediT authorship contribution statement

Lisa Zimmermann: Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing. **Sarah Göttlich:** Formal analysis, Writing - review & editing. **Jörg Oehlmann:** Conceptualization, Writing - review & editing. **Martin Wagner:** Conceptualization, Writing - review & editing. **Carolin Völker:** Conceptualization, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115392.

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