

Same but different: The response to petroleum and biodegradable bio-based microplastics deviates between *Daphnia magna* clones

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ABSTRACT

Microplastics (MP) derived from commodity plastics are considered a threat to ecosystems. Bio-based and biodegradable (BB-) plastics are proposed as eco-friendly alternatives to petroleum-based (PB-) plastics. However, studies on the effects of MP on the model organism *Daphnia magna* often report a wide range of effects for both PB- and BB-plastics. While different physical and chemical MP properties may contribute, other factors, such as differences in clonal sensitivities, are rarely considered. Additionally, only a few studies included a particle control to differentiate between effects caused by particles themselves and those specifically by MP. In this study, these knowledge gaps are addressed by (1) comparing the effects of PB-MP (PET) and two BB-MP (PBS, PLA) on the life-history and morphology of *D. magna*, and (2) examining the responses of two *D. magna* clones to chronic MP exposure (all MP < 20 µm), always including cellulose as a particle control, in a setup with reduced food availability to mimic real environmental scenarios. When comparing PB- to BB-MP, the latter caused similar adverse effects on survival and sublethal life-history traits, while cellulose had no effect or positive effects. We observed a similar concentration-dependent increase in mortality for both clones while data on sublethal parameters were significantly different between the clones. We conclude that particle controls and genetic variability are crucial parameters that should be considered in *D. magna* experiments and that MP effects need to be investigated under environmentally relevant conditions.

1. Introduction

Due to extensive press coverage over the past 20 years, the public

perception of microplastics (MP, 1–1000 µm; (ISO 24187:2023, 2023)) is that they present a significant threat to ecosystems. Meanwhile, scientific interest in the occurrence and ecotoxicological effects of MP has

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grown. MP have been found ubiquitously in the environment; they contaminate the oceans, lakes, rivers, ponds, glaciers, sediments, and soils (Akdogan and Guven, 2019). MP not only interact with and alter different environmental matrices but also have various impacts on organisms across different trophic levels (e.g., Bhatt and Chauhan, 2023).

Given the accumulation of and impacts on the environment from conventional petroleum-based (PB-) plastics such as polypropylene (PP), polyethylene (PE), polystyrene (PS), polyethylene terephthalate (PET), and polyvinyl chloride (PVC) (PlasticsEurope, 2024), the demand for alternatives such as biodegradable and/or bio-based (BB) plastics has increased (Shruti and Kutralam-Muniasamy, 2019). Biodegradable plastics are introduced as a more environmentally friendly alternative to traditional non-degradable plastics. These materials can be petroleum-based or entirely or partly made from renewable resources (bio-based). Due to their presumed lower environmental impacts compared to conventional plastics, market demand for them has grown (Shruti and Kutralam-Muniasamy, 2019). Whether bio-based or not, biodegradable plastics are designed to decompose under suitable environmental conditions (Savva et al., 2023). However, recent research shows that these materials may not fully degrade in nature (Lara-Topete et al., 2024) and can also form persistent MP (Steiner et al., 2022). Among the most produced biodegradable and/or bio-based polymers are polylactic acid (PLA) and polybutylene succinate (PBS) (Haider et al., 2019). OECD (2022) reports that 0.5 % (2.3 Mt) of produced plastics are labeled as “bioplastics”. Global “bioplastic” production is projected to rise from 2.47 Mt in 2024–5.73 Mt within five years (European Bioplastics, 2024). Meanwhile, OECD estimates that overall plastic production, including bioplastics, will triple by 2060, reaching 1230 Mt worldwide (OECD, 2023).

Although plastics are mainly produced and used on land, freshwater environments are among the most seriously impacted ecosystems by plastic pollution (Cuenca Cambronero et al., 2018), including MP. These particles primarily originate from mismanaged plastic waste and littering, urban and agricultural runoff, atmospheric deposition, wastewater, and industrial discharges (Ding et al., 2021). According to the OECD, 109 Mt of plastic accumulated in rivers worldwide in 2019 (OECD, 2022), and MP has been found in lakes and reservoirs worldwide (OECD, 2022). The behavior and transport processes of MP in freshwater systems have been extensively studied in recent years and are highly dependent on hydrodynamics, the particles' physicochemical properties (such as size, shape, surface charge), and the possible interactions with the organic matter present (Guo et al., 2024). Therefore, freshwater ecosystems and their organisms are the focus of numerous studies on MP pollution (e.g., O'Connor et al., 2020; Brehm et al., 2022). When examining the effects of contaminants on freshwater biota, Cladocera, especially *Daphnia* species, are key organisms in most lentic freshwater systems and are well-established model organisms in ecotoxicology and risk assessment (Sarma and Nandini, 2006). Their advantages include sensitivity to various toxicants, short generation times, and rapid population growth through amictic parthenogenesis (Sarma and Nandini, 2006). Parthenogenetic reproduction is often highly valued because it allows researchers to exclude genetic variation when interpreting results (Guan and Wang, 2006). *Daphnia magna* is a preferred species within the genus *Daphnia* for ecotoxicological studies, especially those focused on MP (Brehm et al., 2023). Despite using this clonal model organism, studies on MP toxicity in *D. magna* often produce inconsistent results (e.g., Brehm et al., 2023). A major reason for this variability is the wide range of MP used across studies, which have different physicochemical properties such as plastic-associated chemicals, surface roughness, shape, or surface charge (Wagner et al., 2024; Ali et al., 2024). A recent study on mammalian cell lines emphasized the importance of considering physicochemical properties when interpreting microplastic toxicity studies. Exposure to seemingly identical MP from different manufacturers, all made of the same polymer, within the same size class and with the same shape, elicited different effects (Wieland et al., 2024). Additionally, observed effects could be solely due to the presence of

particles in the gastrointestinal tract of exposed organisms, highlighting the need for appropriate particle controls in MP ecotoxicological research (Rozman and Kalčíková 2022).

In addition to the various physicochemical properties of MP, different clonal sensitivities of *D. magna* strains to environmental stressors may hinder the overall comparability of studies. Important ecotoxicological endpoints include life-history traits (Barata et al., 2000), which vary between *Daphnia* clones due to genetic differences. Typically, researchers conduct experiments using in-house *Daphnia* cultures, consisting of a single clone obtained either from wild populations or other husbandries (e.g., Baird et al., 1991). As a result, studies on *Daphnia* can yield significantly different outcomes, even when using standardized methods, due to the use of different clones. Only a few studies have directly compared the effects of MP on multiple *Daphnia* clones of the same species (Imhof et al., 2017; Sadler et al., 2019; de Souza Machado et al., 2023; Brehm et al., 2023), highlighting the importance of considering genetic variation in MP toxicity experiments.

To fill these knowledge gaps, two *D. magna* clones (Aig and BL2.2) originating from different habitats were chronically exposed to MP in comparison to a particle control. The selected clones are known to respond with different life-history strategies to predation stress, as they originate from different locations. We exposed the two *D. magna* clones to irregular MP (< 20 µm), which was made of PBS, PLA (both biodegradable and bio-based, BB-MP), or PET (petroleum-based, PB-MP). Additionally, microcrystalline cellulose fragments were included as particle control (alongside the blank control) to ensure that a mere particle effect did not cause the observed results. Cellulose was chosen as a control particle because it is a naturally occurring polymer with a density similar to that of the MP used.

This study is the first to directly examine the potential toxicity of the BB-MP PBS on *D. magna*, and compare it with another BB-MP, PLA, as well as with a PB-MP, PET. It is also the first to explore this aspect in the context of clonal sensitivity and an environmentally relevant experimental setup. Such a setup with a lower food supply was selected because feeding *ad libitum* might reduce MP effects (Ogonowski et al., 2016; Schür et al., 2020). However, the chosen food concentration still allows the organisms to grow and reproduce (Lampert, 1978; Schür et al., 2020).

2. Materials and Methods

2.1. *Daphnia magna* cultivation

Two *D. magna* clones from a well-established husbandry were selected for the experiments. The first clone (BL2.2) originated from a pond (Oud Meren) in Leuven (Belgium) and has been maintained in husbandry since 1997 (Imhof et al., 2017). BL2.2 was chosen to enable comparability across studies using the same clone (e.g., Imhof et al., 2017; Schwarzer et al., 2022). The second clone (Aig) was collected in October 2021 from a fishpond of a riverine area called “Aischgrund”, located north-west of Nuremberg (49.657554 N, 10.848952 E), Germany, and has been in culture in our lab since then. The Aig clone was selected because of its different geographical origin, which might translate into different responses to the same environmental stressor (i.e., MP) (Soares et al., 1992). Test organisms used were randomly selected from the second brood of age-synchronized mothers held in 1.5 L glass beakers (Weck, Wehr-Öflingen, Germany). All animals were maintained in M4 medium (Elennd and Bias 1990) with the addition of 4.48 µg L⁻¹ of selenium dioxide (SeO₂) and kept at 20 ± 0.5 °C in a climate chamber with a photoperiod of 14:9 h light: darkness with 30 min of dusk and dawn. The *D. magna* culture was fed *ad libitum* (2 mg C L⁻¹) with the green algae *Acutodesmus obliquus* (SAG 276–3a, Sammlung von Algenkulturen, Göttingen, Germany). The mothers were separated from the offspring (< 24 h) to ensure the use of similarly aged daphnids for the experiments.

2.2. *Acutodesmus obliquus* culture

The green algae *A. obliquus* were cultivated in-house. Algae cultures were maintained in 2.5 L Erlenmeyer flasks, equipped with a filtered aeration system. Each flask was filled with 1.5 L of Z medium (Prusch and Minck, 1985) and 500 mL of *A. obliquus* batch. The glassware, aeration tubes, and Z medium were sterilized before setting up a new culture. The so-prepared cultivation was maintained in a climate chamber at a temperature of 21 ± 0.5 °C, with a photoperiod of 16: 8 h light: darkness.

2.3. Microplastics and cellulose particle production and characterization

Three types of MP and one type of cellulose fragments (< 20 µm) were used in this study. Two plastics were biodegradable and bio-based (BB-): PBS (BioPBS FZ91PM, Mitsubishi Chemicals, Japan; molecular weight (Mw) = 2.19×10^5 g mol⁻¹) and PLA (PDLLA, NatureWorks, USA; Mw = 1.95×10^5 g mol⁻¹), and one was a petroleum-based (PB-): PET (NEOGROUP, Lithuania; Mw = 9.77×10^4 g mol⁻¹). We used microcrystalline cellulose fragments as non-plastic control particles (CAS: 9004-34-6, Sigma Aldrich, Germany).

While the plastic fragments were produced in-house by milling and sieving, the purchased cellulose fragments were used as received to prepare the particle stock suspensions, which are described below. The following outlines the required steps for producing the employed MP fragments: As a base material, 1 kg of polymer granulate was mixed with liquid N₂ for 10 min and then milled using an ultra-centrifugal mill (ZM 200, Retsch, Germany, 18000 min⁻¹, 1000 µm spacer sieve, 24 tooth rotor). The ground polymer was then dried under a high vacuum at 40 °C for 24 h. The 1000 µm polymer fragments were first mixed with shredded dry ice (1:1 V/V) and then with liquid N₂ for 20 min. This mixture was poured into the ultra-centrifugal mill (approx. 10 min, 18000 min⁻¹, 200 µm spacer sieve, 24 tooth rotor). Subsequently, the particles were dried as described above and sieved using an ALPINE Air Jet sieve e200LS machine (Fritsch, Germany) with a 75 µm sieve (20 min, 2000 Pa). The fraction < 75 µm was again sieved using a 20 µm sieve (20 min, 2000 Pa), and the obtained particles (< 20 µm) were used for experiments. The particle size distributions were determined via a combination of laser diffraction and dynamic image analysis (Figure SI 1, FLOWSYNC, Microtrac MRB, Montgomeryville and York, USA).

For particle characterization, gel permeation chromatography (GPC) and nuclear magnetic resonance spectroscopy (NMR) were performed. GPC was used to measure molecular weight (MW) on a 1260 Infinity system (Agilent Technologies). The instrument was fitted with a pre-column SDV 5 µm, the main column SDV linear XL 5 µm (PSS, Mainz, Germany), and a refractive index detector (RI, Agilent Technologies, Figure SI 2). These measurements were conducted on 2–3 mg of each polymer. ¹H (300 MHz) NMR spectra were recorded on an Ultrashield-300 spectrometer (Bruker) in CDCl₃, and the residual peak from the CDCl₃ was used as an internal standard. These measurements were conducted on 15–20 mg of plastics.

Scanning electron micrographs of the MP and cellulose particles were obtained using a JEOL JSM-IT500 Scanning Electron Microscope (SEM, JEOL Ltd., Japan). Before imaging, particles were placed on conductive carbon pads (G3347, Plano GmbH, Wetzlar, Germany) and attached to aluminum stubs (Ødiameter 12 mm, Plano GmbH, Wetzlar, Germany). The samples were then coated with 2 nm of carbon and 2 nm of platinum (Leica EM ACE600, Leica Microsystems GmbH, Germany).

Before the start of the experiment, particle suspensions were prepared by adding 100 mg of each particle type (MP and cellulose) to 100 mL of autoclaved M4 medium. Before use, stock suspensions were shaken for 48 h at 100 rpm. Particle counting was conducted at an optical microscope (Zeiss Axio Lab.A1, Carl Zeiss Microscopy GmbH, Jena, Germany) and repeated three times using a hemocytometer (Neubauer Improved, Brand, Germany) to determine the average particle concentration in the stock suspensions.

To assess the potential digestion of cellulose particles, fecal pellets from *D. magna* individuals were collected in a separate experimental setup (one animal in a 160 mL Weck beaker, replicated three times, 5000 particles mL⁻¹ concentration) and measured using µFT-IR spectroscopy. To collect the fecal pellets, the animals were transferred onto a microscopic slide after 24 h of incubation (without food), and observed under a stereo microscope (Leica MS5, Leica Microsystems, Wetzlar, Germany). The fecal pellets were collected immediately after being released by the individual daphnid and then promptly transferred onto an Anodisc 25 (0.2 µm pore size, 25 mm diameter; Product number: 6809–6022, Whatman, Germany). The collected samples were dried overnight at room temperature inside a petri dish. Spectra were obtained using a Lumos II µFT-IR microscope (Bruker Corporation, USA).

2.4. Chronic toxicity test

A 21-day chronic toxicity experiment was conducted following OECD Guideline 211 (Test No. 211: *Daphnia magna* Reproduction Test, 2008) with a few modifications (Fig. 1).

The animals were exposed under semi-static conditions to two particle concentrations, selected based on Schwarzer et al. (2022): 500 and 5000 particles mL⁻¹. These concentrations were selected to allow the detection of potential concentration-dependent effects, and are therefore higher than those found in freshwater environments (Gupta et al., 2023). As a control, we exposed the organisms to cellulose particles at the same concentrations. Each treatment included 15 replicates, meaning 160 mL Weck beakers containing 50 mL of modified M4 medium and one individual daphnid. A blank control without added particles was included for both clones. However, for analysis, it was decided to use the cellulose exposure group as a control for comparison, to distinguish between effects attributable to particles in general and those attributable to MP. Therefore, in this manuscript, the blank control will be referred to as “concentration 0” (0 particles mL⁻¹) and will be considered a “particle concentration.”

To ensure consistent exposure conditions, the medium, including the particles, was renewed every other day. Daphnids were fed 0.5 mg C L⁻¹ (0.025 mg C per individual) of *A. obliquus*, provided to the animals with the renewed medium. Most recent studies offer food *ad libitum* (i.e., 1.5–2 mg C L⁻¹), a scenario that rarely occurs in nature. In this work, the term “reduced food availability” refers to a food supply lower than the amount recommended by the OECD guidelines for testing on *D. magna* (OECD No. 211), aiming to better reflect actual environmental conditions.

Every other day, pH and O₂ levels were measured, showing only minor differences between treatments (mean values and respective standard deviations: BL2.2: pH: 7.59 ± 0.49 , O₂: 8.11 ± 0.14 mg L⁻¹; Aig: pH: 7.57 ± 0.51 , O₂: 8.18 ± 0.17 mg L⁻¹).

Different parameters were recorded at primiparity (when the first clutch of eggs appears in the *Daphnia* brood chamber) and at the end of the exposure. Specifically, during the 21-day exposure, we recorded mortality on a daily basis, body length at primiparity and at the end of exposure, age at primiparity, the daily number of hatched neonates, and the sex of the neonates. For analysis, the body length at primiparity was normalized based on the number of days needed for each individual to reach that stage. Images for body length analysis were taken using a dissecting microscope equipped with a digital camera (Leica M50, Germany; camera: Olympus DP26, Germany; light: Leica KL 300 LED, Germany). Body length was measured from the top of the compound eye to the base of the spine (Figure SI 3) with the software cellSens Dimension (v1.11, Olympus, Germany). The newly hatched neonates were collected and reared separately for five days, allowing for visual sex determination.

2.5. Statistical analysis

The statistical analyses were conducted using R version 4.2.2 (R Core

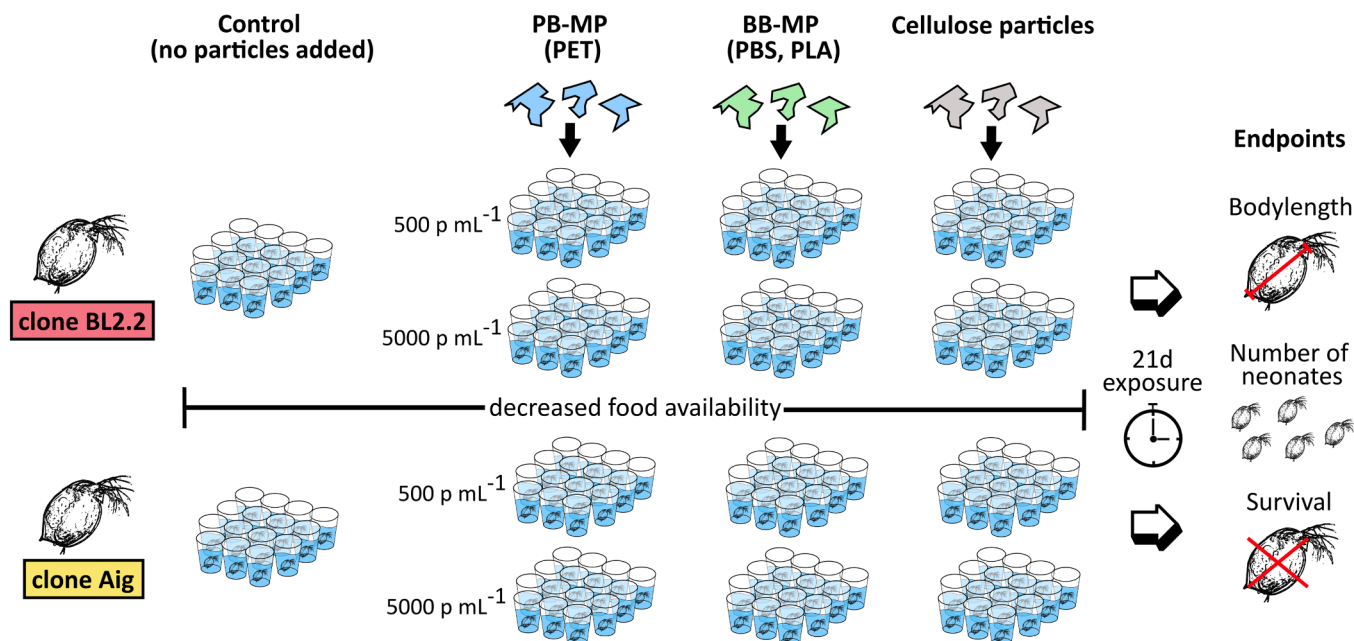


Fig. 1. Schematic depiction of the experimental setup ($n = 15$ per treatment at $t = 0$).

Team, 2022). Regression models (ANOVA) were applied to analyze the data. A linear regression model was employed for morphological parameters and reproduction data, while a logistic regression model, using a logarithmic scale for concentration, was used to analyze survival. Normality and homogeneity of variance of residuals were tested as prerequisites for ANOVA. Each response parameter was analyzed with a three-way interaction between treatment, particle concentration, and clone. Significant models were subsequently analyzed with pairwise comparisons using Tukey HSD post hoc tests and Holm's correction method for multiple comparisons. Furthermore, Cohen's D effect size was calculated. The package car version 3.1.1 (Fox and Weisberg, 2018) was used for ANOVA, emmeans version 1.8.2 (Lenth et al., 2020) for post hoc tests, and DHARMA version 0.4.6 (Hartig, 2022) to validate the logistic regression models used in the survival analyses. Dead individuals were excluded from analyses of sublethal endpoints. Juvenile body length growth rate was included as a parameter to account for the

number of days each individual took to reach sexual maturity, improving data comparability.

The two clones may respond differently to the treatments, but they also have an inherent difference (i.e., their size already varies under control conditions). To distinguish the inherent clone difference from the variation in response to the treatment, we additionally centered the values of all individuals for each considered sublethal parameter (juvenile body length growth rate, body length, number of neonates) to the respective test concentration 0 (further details and results are in the SI).

3. Results

3.1. Particle characterization

The identity of the employed polymers was confirmed via H NMR (Fig. 2). The respective MW analyses conducted via GPC are reported in

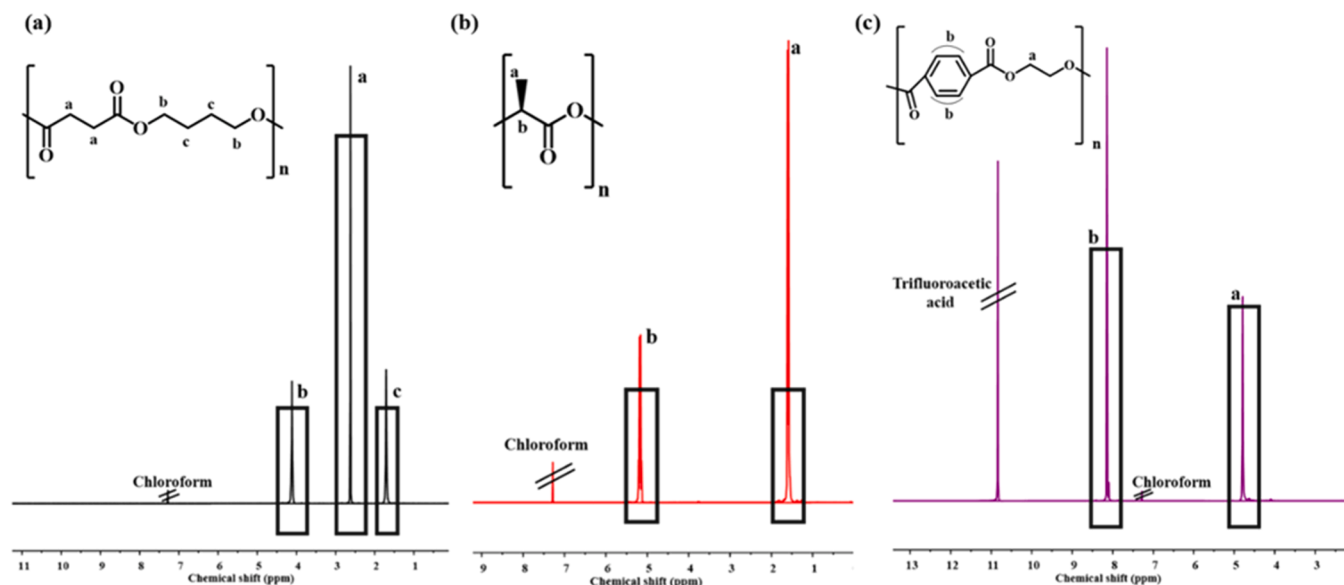


Fig. 2. ¹H NMR (300 MHz) spectra of a) PBS, b) PLA, and c) PET in CDCl₃.

the SI (Figure SI 2).

Size characterization was conducted for each particle type, including those milled in-house and the purchased cellulose fragments. We selected particles with similar d_{50} values: cellulose $d_{50} = 25.65 \mu\text{m}$, PBS $d_{50} = 22.45 \mu\text{m}$, PLA $d_{50} = 21.25 \mu\text{m}$, and PET $d_{50} = 21.82 \mu\text{m}$. Size distribution charts are shown in the SI (Figure SI 1).

SEM analysis revealed that all MP particles (PBS, PLA, PET) and the particle control (cellulose) were similar in shape and size (Fig. 3).

3.2. Survival

No mortality was observed at concentration 0 (no particles added) and the cellulose treatment at any of the applied concentrations (Table SI 1). On the other hand, all MP treatments (BB- and PB-) showed a negative effect on survival ($\chi^2_{(3, 341)} = 56.3$, $p < 0.001$) that increased at the higher MP concentration of 5000 p mL^{-1} ($\chi^2_{(3, 341)} = 85.2$, $p < 0.001$) (Fig. 4 and Figure SI 4). PBS had the highest mortality in both clones, followed by PET and PLA. At the highest PBS concentration (5000 p mL^{-1}), only one out of 15 individuals (6.67 %) survived in the case of the Aig clone, and four (26.67 %) survived in the BL2.2 clone after 21 days of exposure. The lower PBS concentration (500 p mL^{-1}) also induced mortality in BL2.2 clones, with only five (33.33 %) animals surviving at the end of the exposure (Table SI 1). At the highest PET concentration (5000 p mL^{-1}), only five (33.33 %) individuals survived across both clones. The highest concentration of PLA (5000 p mL^{-1}) also caused mortality, albeit to a lesser extent, with ten (66.67 %) and nine (60 %) individuals surviving for the Aig and BL2.2 clones, respectively. Analysis of the survival data revealed no clonal dependency (Table 1 & 2), as mortality rates were similar between the two clones.

3.3. Reproduction

The analyses conducted on the average number of neonates indicated a significant effect of the clone ($F_{(3263)} = 172.5$, $p < 0.001$) and an interaction between the treatment and the particle concentration ($F_{(3263)} = 3.50$, $p = 0.015$). The cellulose particles showed neutral to positive effects (not significant), while the MP showed significant adverse effects. The interaction between the treatment and the clone ($F_{(3263)} = 2.96$, $p = 0.032$) and the interaction between particle concentration and clone ($F_{(3263)} = 9.17$, $p = 0.002$) were also significant.

Exposure to MP (both BB- and PB-) in the BL2.2 clone caused a concentration-dependent reduction in the reproductive output from the concentration 0 compared to the organisms exposed to cellulose, which produced a steady number of offspring regardless of the concentration. In contrast, the Aig clone had a generally low reproductive output across the selected treatments, including the concentration 0 and the cellulose control (Table SI 2).

In the BL2.2 clone, a moderate effect caused by all MP treatments (BB- and PB-) was observed (effect size in Table 1). This effect decreased with concentration. For the Aig clone (also confirmed by the effect size analyses, Table 1), the number of neonates produced was generally lower. As a result, no differences between the treatments were observed. In the BL2.2 clone, PBS and PET caused significant decreases in neonate numbers (Fig. 5 and Figure SI 5). All hatched neonates from both clones were female. Clonal comparison revealed significant differences between the two clones for each treatment (Table 2).

3.4. Juvenile growth rate

Analysis of juvenile body length growth rate showed that the exposed organisms were significantly affected by the treatment ($F_{(3299)}$

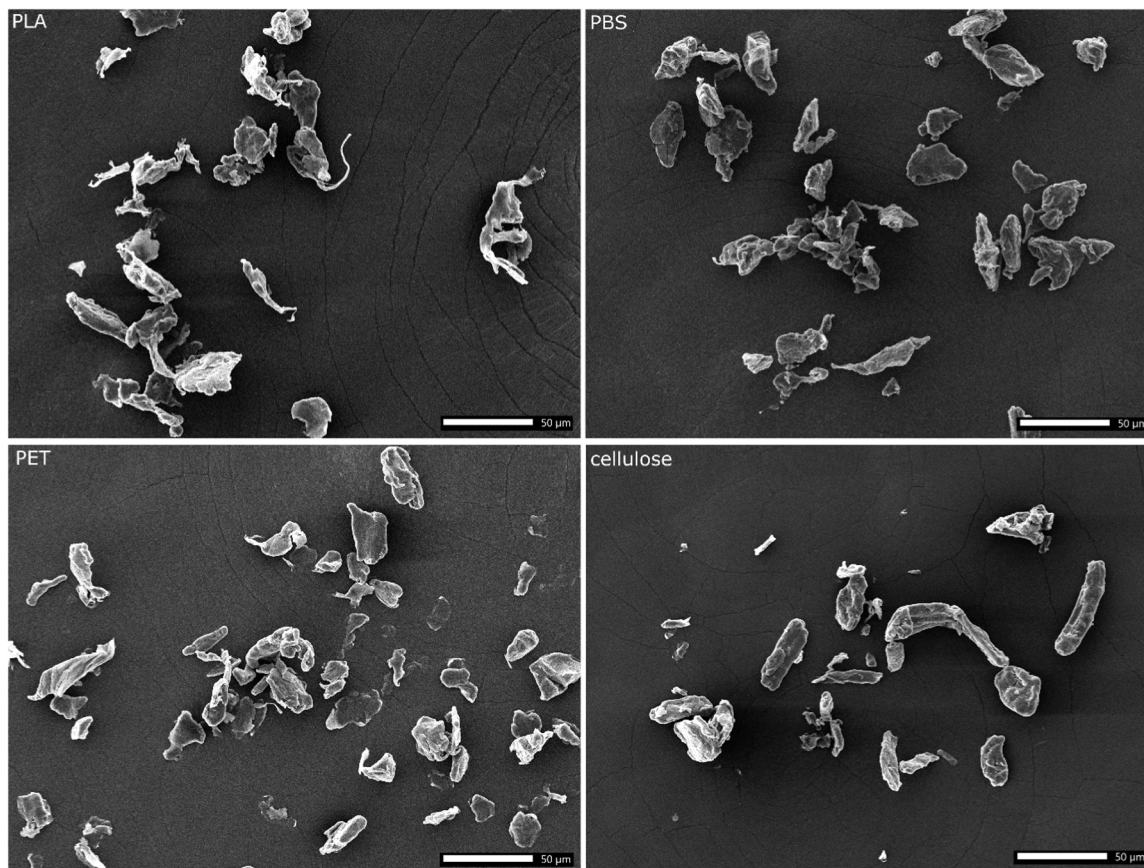


Fig. 3. SEM micrographs of MP and cellulose particles. 400x magnification, scale bars = 50 μm .

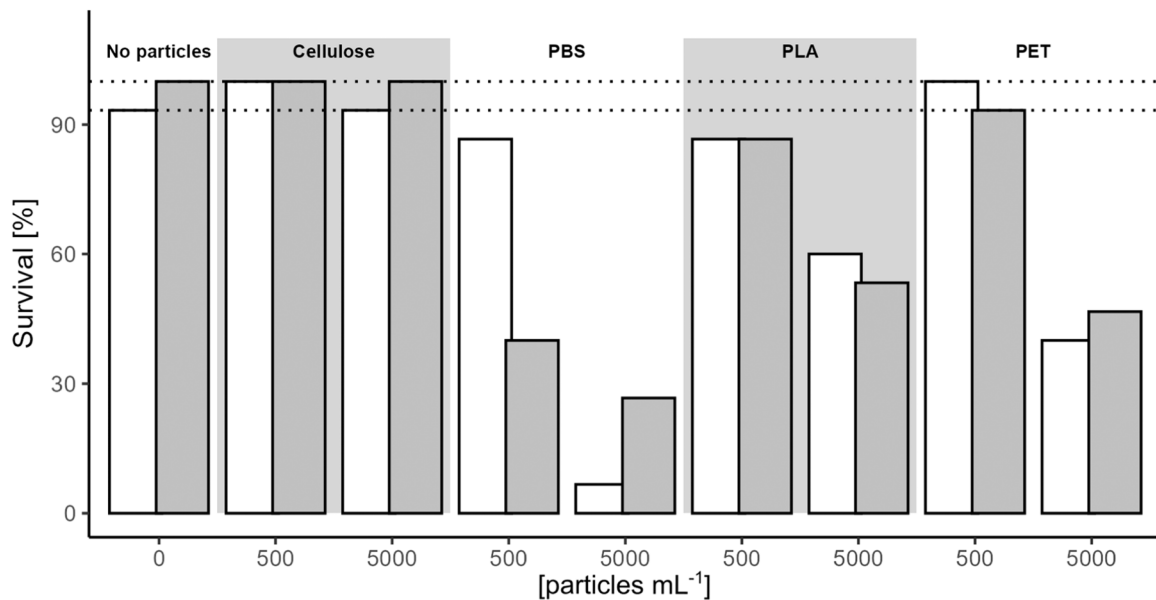


Fig. 4. Percentage of surviving *D. magna* individuals at the end of exposure ($n = 15$ per treatment at $t = 0$) for the BL2.2 clone (grey bars) and the Aig clone (white bars), exposed to PET, PBS, and PLA MP, or cellulose fragments. The dashed lines show the value of the blank control ($0 \text{ particles mL}^{-1}$).

Table 1

Pairwise comparison of the treatments between both clones for life history parameters of *D. magna* exposed to PBS, PLA, and PET MP or cellulose fragments. To indicate effect size, we present z-values for survival and t-values for the other traits. Bold values represent significance and a detectable effect ($p < 0.05$ and $D > 0.3$).

Aig vs BL2.2				
Response	Treatment	t/z value	P value	D
Survival	cellulose	-0.01	0.98	-18.8
	PBS	0.98	0.32	1.12
	PLA	-1.15	0.25	-1.76
	PET	-1.36	0.17	-3.01
	cellulose	-9.06	< 0.001	-1.99
Num. of neonates	PBS	-3.25	0.001	-1.09
	PLA	-7.04	< 0.001	-1.67
	PET	-4.74	< 0.001	-1.17
	cellulose	-2.81	< 0.01	-2.08
Juvenile growth rate	PBS	-2.06	0.03	-0.49
	PLA	-4.28	< 0.001	-0.96
	PET	-2.23	0.02	-0.50
	cellulose	-6.90	< 0.001	-1.52
Body length (21 d)	PBS	-3.64	< 0.001	-1.22
	PLA	-5.60	< 0.001	-1.33
	PET	-4.09	< 0.001	-1.01

$= 3.75$, $p = 0.011$), particle concentration ($F_{(3299)} = 64.49$, $p < 0.001$), clone ($F_{(3299)} = 41.48$, $p < 0.001$), and the interaction between treatment and particle concentration ($F_{(3299)} = 4.15$, $p < 0.001$).

The individuals in all MP treatments (BB- and PB-) grew slightly slower compared to the respective cellulose control treatment when exposed to $5000 \text{ particles mL}^{-1}$, while those exposed to $500 \text{ particles mL}^{-1}$ had a growth rate comparable to both the cellulose control and the concentration 0. Surprisingly, regarding the BL2.2 clone, the animals grew faster when exposed to cellulose, especially at the higher concentration. Here, significant differences between animals exposed to cellulose were found. A concentration-dependent decrease was also observed for the Aig clone, in this case also in the organisms exposed to cellulose. Pairwise comparisons of the treatments within the separate clones indicate significant differences only between PBS and PLA ($t = 2.74$, $p = 0.038$) for the Aig clone, with the organisms exposed to PBS being larger than those exposed to PLA (Fig. 6, Figure SI 6, and Table SI 2). The clonal comparison indicates significant differences

across all treatments, also supported by the corresponding effect sizes (Table 2). Further data regarding the age of primiparity and body length at primiparity are reported in the SI (Figure SI 7 and 8, Table SI 2–4).

3.5. Body length after 21 days of exposure

Analysis of the body length measurements of the test animals recorded on day 21 (Fig. 7 and Figure SI 9) indicated a significant effect of the treatment ($F_{(3263)} = 5.8$, $p < 0.001$), the particle concentration ($F_{(3263)} = 77.4$, $p < 0.001$), the clone ($F_{(3263)} = 122.7$, $p < 0.001$), and the interaction between treatment and particle concentration ($F_{(3263)} = 11.2$, $p < 0.001$).

Individuals from the BL2.2 clone generally grew larger than those from the Aig clone, which was already clear at concentration 0 (Table SI 2). The two clones were confirmed to be significantly different through clonal comparison analyses (Table 2). When examining the clones individually, the only significant difference was found for the treatment with cellulose and PLA for the BL2.2 clone (Table 1). We did not observe concentration-dependent differences in organisms exposed to cellulose, as their sizes remained steady. However, a decrease in size at higher MP concentrations was observed. Concerning the Aig clone, we observed an overall size decrease at high concentrations, regardless of the treatment, including cellulose. Analysis of effect sizes showed a low effect for the Aig clone exposed to both BB-MP (PBS and PLA), while a medium effect was seen in the BL2.2 clone across all MP treatments (Table 1).

4. Discussion

In this study, we examined the effects of PB-MP (PET) and two BB-MP (PBS and PLA) compared to cellulose fragments as a particle control on two *D. magna* clones to assess interspecific sensitivity. Polymer identity was confirmed via H NMR spectroscopy (Fig. 1), and size analyses confirmed the use of similar-sized particles with a $d_{50} \sim 20 \mu\text{m}$ (Figure SI 1). BB-MP had significant effects on the life history of *D. magna* that were similar to, if not stronger than, those of conventional PET-MP, while cellulose control particles had no or slightly positive effects. This suggests that the observed effects cannot be attributed solely to particle exposure. In cellulose-exposed animals, mortality was nearly absent. In the MP treatments, on the other hand, increased mortality rates with higher MP concentrations were found, being similar

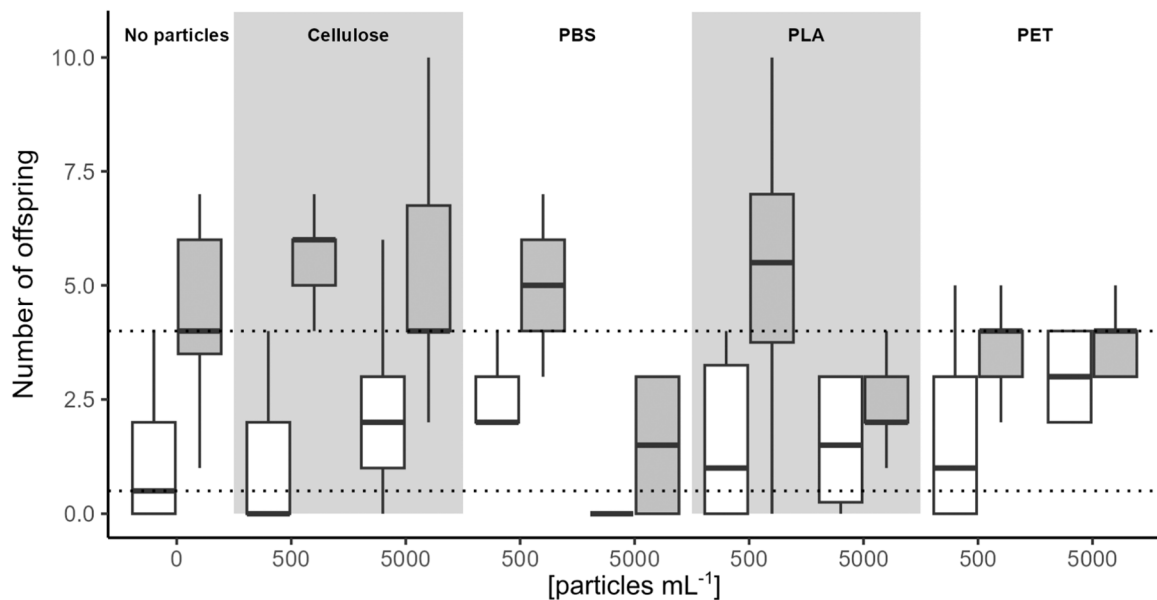


Fig. 5. Cumulative number of neonates from *D. magna* individuals of the BL2.2 clone (grey boxes) and the Aig clone (white boxes) hatched during chronic exposure to PET, PBS, and PLA MP or cellulose fragments. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. The whiskers represent the 1.5-fold of the reported quartiles. The dashed lines report the median value of the blank control (0 particles mL⁻¹).

Table 2

Pairwise comparison between the separate clones of the life history parameters of *D. magna* exposed to PBS, PLA, and PET MP or cellulose fragments. To indicate effect size, we present z-values for survival and t-values for the other traits. Bold values represent significance and a detectable effect ($p < 0.05$ and $D > 0.3$).

Response	Comparison	BL2.2			Aig		
		t/z value	P value	D	t/z value	P value	D
Survival	Cellulose-PBS	0.02	1.000	21.75	1.41	0.791	1.82
	Cellulose-PLA	0.02	1.000	19.04	1.88	0.354	1.99
	Cellulose-PET	0.01	1.000	17.81	1.00	1.000	1.27
	PBS-PLA	-1.74	0.410	-2.70	0.15	1.000	0.17
	PBS-PET	-2.20	0.165	-4.66	-0.41	1.000	-0.54
	PLA-PET	-1.61	1.000	-1.96	-0.66	1.000	-0.71
Num. of neonates	Cellulose-PBS	2.65	0.041	0.68	-0.70	1.000	-0.21
	Cellulose-PLA	1.62	0.418	0.37	0.20	1.000	0.04
	Cellulose-PET	2.78	0.034	0.65	-0.71	1.000	-0.17
	PBS-PLA	-1.19	0.703	-0.31	0.84	1.000	0.26
	PBS-PET	-0.14	0.887	-0.03	0.15	1.000	0.04
	PLA-PET	1.15	0.703	0.27	-0.89	1.000	-0.21
Juvenile growth rate	Cellulose-PBS	2.21	0.108	0.50	-0.54	1.000	-0.12
	Cellulose-PLA	2.96	0.016	0.65	2.33	0.100	0.52
	Cellulose-PET	3.16	0.010	0.70	0.53	1.000	0.11
	PBS-PLA	0.68	1.000	0.15	2.74	0.038	1.11
	PBS-PET	0.88	1.000	0.20	1.04	0.899	0.70
	PLA-PET	0.21	1.000	0.05	-1.80	0.290	0.03
Body length (21 d)	Cellulose-PBS	2.32	0.084	0.60	0.99	1.000	0.3
	Cellulose-PLA	2.70	0.044	0.61	1.85	0.387	0.42
	Cellulose-PET	2.59	0.050	0.60	0.43	0.436	0.1
	PBS-PLA	0.05	1.000	0.01	0.39	1.000	0.12
	PBS-PET	0.01	1.000	0.003	-0.64	1.000	-0.20
	PLA-PET	-0.04	1.000	-0.009	-1.33	0.915	-0.32

across both clones. Life-history analyses and morphological parameters showed different sensitivities between clones.

4.1. Effects of MP on *D. magna*'s life history

BB-MP generally were at least as toxic to the *D. magna* clones (PBS > PLA) as PET-MP. In this study, we included cellulose as a non-plastic control particle to rule out a mere particle effect on the daphnids. Our results showed that cellulose particles had no effect or slightly positive effects on the selected sublethal parameters, which was also previously reported by Schwarzer and colleagues (2022). Therefore, the effects observed from MP are likely caused by the properties of the different MP

tested rather than by general particle exposure. In this context, other studies suggest that the effects of MP may be exacerbated by reduced food intake resulting from the presence of MP in the gastrointestinal system (e.g., Höss et al., 2022). The use of microcrystalline cellulose as a control particle demonstrates that, despite providing less food, the mere presence of particles does not explain the adverse effects observed in this study. The experimental setup, which involved reduced food availability and MP exposure, also significantly affected the Aig clone, making it impossible to isolate the specific effects of MP on reproduction for this clone.

Several recent studies also found adverse effects of BB-MP on aquatic organisms, especially of MP made of PLA and PHB (Zimmermann et al.,

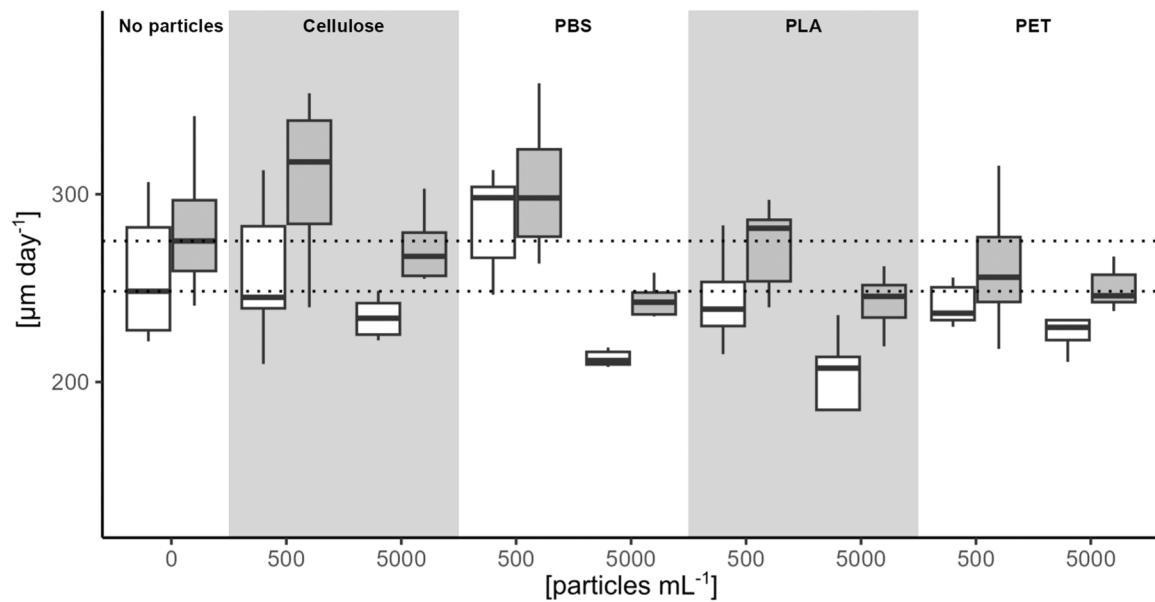


Fig. 6. Juvenile growth rate ($\mu\text{m day}^{-1}$) of *D. magna* from the BL2.2 clone (grey boxes) and the Aig clone (white boxes) exposed to PET, PBS, and PLA MP or cellulose fragments. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. The whiskers represent the 1.5-fold of the reported quartiles. The dashed lines report the median value of the blank control (0 particles mL^{-1}).

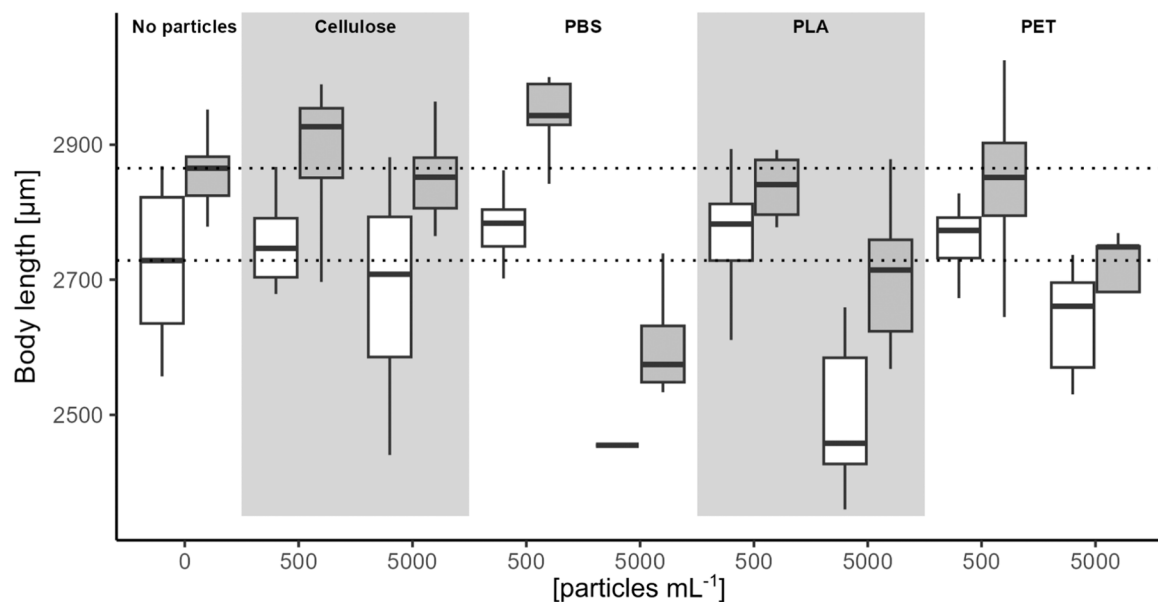


Fig. 7. Body length at the end of chronic exposure of *D. magna* individuals from the BL2.2 clone (grey boxes) and the Aig clone (white boxes) exposed to PET, PBS, and PLA MP or cellulose fragments. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. The whiskers represent the 1.5-fold of the reported quartiles. The dashed lines report the median value of the blank control (0 particles mL^{-1}).

2020; An et al., 2024; De Felice et al., 2024). For instance, Zimmermann and co-authors (2020) demonstrated, in a similar experimental setup, that PLA-MP exerted effects comparable to those of conventional MP (PVC and PUR) when compared to kaolin as a particle control. The experiment, conducted across a wide concentration range (10–500 mg L^{-1}), showed a concentration-dependent decrease in both the number of offspring produced and the mean body length for all MP types. Notably, PLA exposure was associated with a significant increase in mortality. Similarly, a study by An et al. (2024) found that PLA exhibited greater toxicity than PET-MP in a 21-day chronic exposure test. Compared to PET, PLA resulted in a reduction in survival rate and fitness, accompanied by the downregulation of genes related to reproduction, oxidative stress defense, and cellular energy production.

Likewise, De Felice et al. (2024) reported that PLA altered biomarkers associated with oxidative stress and energy metabolism, as well as the swimming activity of *Daphnia* after 21 days of exposure.

These studies collectively highlight that PLA, despite being marketed as an environmentally friendly alternative, is capable of inducing significant adverse effects in *Daphnia*, comparable to or even exceeding those caused by PB-MP.

Concerning PBS, recent scientific literature has not yet provided studies on its impact on *Daphnia*. Thus, the present study represents the first report on PBS toxicity on *Daphnia*. In our study, we found that PBS was more toxic than PLA or PET. Despite these findings, other research reports very different outcomes when comparing PB- and BB-MP. For example, a study by Brehm et al. (2022) on *Dreissena bugensis* reported

that MP from recycled PET had stronger adverse effects than PLA-MP. These differences in assessing the toxicity of the same MP may be due to the diverse physicochemical properties of the particles used, mainly related to leaching chemicals (Zimmermann et al., 2020; Brehm et al., 2022; An et al., 2024), which, in the case of Brehm et al. (2022), likely accumulated in the recycled material. Similarly, a more recent study reported stronger adverse effects of PET compared to BB-MP derived from PHB (Serra et al., 2025). Since the chemical composition of the same plastic type varies based on their final properties, products made from the same polymer can have very different chemical compositions (Laforsch et al., 2020). The chemicals used in plastic manufacturing are largely unknown because producers are not required to disclose the compounds they use (Wagner et al., 2024). To date, over 16,000 plastic chemicals have been identified; about 4200 of these are of concern for environmental and human health because they are classified as persistent, bioaccumulative, mobile, and/or toxic (Wagner et al., 2024). MP toxicity is, in addition to their potential hazardous chemical compositions, also influenced by their shape and size (Schwarzer et al., 2022). In this study, however, these factors can largely be excluded, as the morphology of the particles used appeared to be very similar (Fig. 2).

While most chronic MP toxicity studies using *Daphnia* as a model organism are conducted with *ad libitum* food (e.g., Brehm et al., 2023), we performed our experiment using a more environmentally relevant setup. A high amount of added food in an experiment could reduce the potentially harmful effects of MP particles, as excessive food intake may lower particle uptake and limit contact with the gastrointestinal tract, making MP effects harder to detect (Schür et al., 2020; Lyu et al., 2022). This method of conducting experiments with *ad libitum* food is recommended by OECD guidelines for *D. magna* experiments (OECD No 211). However, this protocol was originally designed for testing soluble chemicals that interact with the entire body surface, regardless of food levels. Particulate contaminants, such as MP, which are mixed with food particles potentially compete with each other. Furthermore, feeding *ad libitum* does not accurately mimic natural environments where nutrient levels are lower and fluctuate seasonally (Giebelhausen and Lampert, 2001). Effects on life-history traits are often influenced by high food intake. However, food scarcity is common in natural environments, which means that the potential threat posed by MP may be significantly underestimated in experiments that feed animals *ad libitum* (Lyu et al., 2022). Therefore, in our study, animals were fed 0.5 mg C L^{-1} , a realistic food amount for mesotrophic lakes that still supports growth (Giebelhausen and Lampert, 2001) and egg production in *Daphnia* ($> 0.2 \text{ mg C L}^{-1}$; Lampert, 1978). This more natural scenario could have influenced the measured endpoints, as the available food concentration is a key factor in *Daphnia*'s growth and reproduction (Lampert and Trubetskova, 1996). As a result, both clones probably responded to the reduced food availability, as indicated by their generally low neonate production. Both clones showed a significantly lower survival rate across all three MP treatments compared to cellulose, suggesting a combined adverse impact of limited food availability and MP exposure. We recognize that confirming this hypothesis requires future studies to explore how food scarcity influences MP effects. Nonetheless, prior research supports this combined effect (Jemec et al., 2016), and similar findings have been observed in mussels, where food particles mitigated MP's adverse effects (Wang et al., 2021). Conversely, Aljaibachi and Callaghan (2018) reported that *D. magna* mortality from chronic MP exposure depended more on food availability than MP concentration. However, in our study, mortality increased with higher MP levels, indicating that MP toxicity is also dose-dependent. To rule out effects from general particle exposure, we used a cellulose control group. An advantage of using cellulose as a control is its similar density to that of most MP (Schwarzer et al., 2022). Nonetheless, *Daphnia* can produce cellulase enzymes and potentially use cellulose as a carbon source (Schoenberg et al., 1984). Therefore, we cannot exclude the possibility that cellulose fragments in the particle control treatment may have partially offset the reduced food availability, especially for the BL2.2

clone, particularly regarding juvenile growth. To examine this, we analyzed *D. magna* fecal pellets (Figure SI 10) containing the cellulose particles using $\mu\text{FT-IR}$ spectroscopy. The spectra (Figure SI 11) showed no significant difference between cellulose in fecal pellets and pristine cellulose. This suggests that, if digestion occurs, it is limited to the outer layers of the particles and does not change their chemistry, size, or appearance. Another aspect to consider is the potential positive effect of nutritious biofilm formation on the surface of the particles, which could provide nutrients to the daphnids (Amariei et al., 2022) and thus counteract the negative effect of reduced food availability in our experimental setup, explaining the neutral to positive outcomes observed in the cellulose-exposed animals. However, further studies are needed to better explore this aspect.

In general, the mechanisms underlying increased MP toxicity when combined with a limited food supply remain not fully understood. However, in environments where food availability is a limiting factor, MP concentrations are usually lower than those used in this study (Yang et al., 2022). Nonetheless, the harmful effects of MP on *Daphnia* in the environment may depend not only on MP concentration but also on MP properties (Zimmermann et al., 2020; Schwarzer et al., 2022). This is evidenced by the fact that all MP used in this study caused adverse sublethal effects compared to cellulose fragments, even at the lower concentration. These sublethal effects of MP might have long-term consequences for aquatic ecosystems (Haney and Rochman, 2025). However, for risk assessment of these factors, lower particle concentrations should be used, as the ones used in this study are substantially higher than those found in the environment (Gupta et al., 2023).

4.2. Effects of clonal variation

The results of our study showed that the chosen *D. magna* clones significantly differed in all the examined sublethal parameters (Table 1). These findings align with previous research (de Souza Machado et al., 2023), which also observed clonal differences across various stressors, such as chemicals (Barata et al., 2002), predation (Weber and Declerck, 1997), food availability (Glazier and Calow, 1992), and temperature (Sadler et al., 2019).

In the present study, the Aig clone was more negatively affected by MP exposure than the BL2.2 clone. The observed differences could be due to inherent genetic differences between the *D. magna* clones, with the Aig clone being more vulnerable to the introduced stressors than BL2.2. For example, the Aig clone may be more sensitive to reduced food availability, which could have contributed to the stronger effects of MP. Additionally, both clones differ in size, with BL2.2 being larger than Aig (Figure SI 12), which may influence their sensitivity to MP-related toxicity. Sensitivity to various contaminants can be affected by the body size of the organisms, a factor that impacts both toxicodynamics and toxicokinetics, determining intra- and interspecific differences (Gergs et al., 2015). Previous research on other contaminants has shown that smaller daphnids tend to be more sensitive in toxicity tests, as they have higher metabolic rates compared to larger organisms (e.g., Vesela and Vijverberg, 2007) and a larger surface-to-volume ratio. The different responses of the clones might be due to adaptations to their respective environments. For instance, the Aig clone originated from a fishpond, where these organisms face greater predation pressure from fish. As a result, they might be smaller to avoid visual-hunting predators (Dodson, 1989). This adaptation to predation risk in their original habitat could have made them more vulnerable to MP and likely other human-made contaminants.

To rule out that the recorded effects could be solely attributed to clone-specific trait differences, it is necessary to consider these traits when comparing the response of the two different clones to MP exposure. Thus, we normalized (centered) the responses of the two clones with their respective controls (more details in the SI, Table SI 5, and Figures SI 12 and 13). This analysis indicated that treatment and MP concentration significantly affected all sublethal endpoints, and that the

clones differed in susceptibility for the endpoints “reproductive output” and “juvenile body length growth rate.” In the case of reproduction, the BL2.2 clone was significantly affected by MP exposure, whereas the Aig clone was not. However, such a comparison is somewhat limited by the generally low reproduction of the latter clone. For juvenile body length growth rate, the difference between the clones was significant, indicating a stronger influence during the juvenile stages. Extended developmental periods coupled with smaller body sizes at sexual maturity, as observed in the Aig clone, may have negative consequences for natural populations, potentially leading to their decline and cascading effects on higher trophic levels.

Overall, our results on clonal differences showed that clearly identifying the *Daphnia* clone used in a study is critically important, especially in MP research, where the material complexity already reduces study comparability. While the number of studies using *Daphnia* as a model organism in MP research is steadily increasing, the clone has only been reported in a few cases (Brehm et al., 2023). More precise and transparent reporting of the clones used in various experiments could improve comparability between laboratories, leading to better risk assessments and, ultimately, a more trustworthy tool for policymakers.

5. Conclusion

This study compared the effects of MP from a PB-MP (PET) and two BB-MP (PBS and PLA) with those of the particle control cellulose on two different *D. magna* clones. Our results showed that the selected BB-MP (PBS and PLA) caused even more negative or similar effects on daphnids compared to the chosen PB-plastic (PET), depending on the endpoint or *Daphnia* clone considered. In contrast, cellulose particles exhibited neutral to positive effects. Further studies should incorporate more MP types and a wider range of concentrations to improve risk assessment, including environmentally relevant concentrations. Nevertheless, additional research on the impacts of BB-plastics is urgently needed before they can be regarded as an environmentally friendly alternative to PB-MP. The data discussed in this study, however, add to the growing amount of evidence that these polymers should not be regarded as such. This is especially important because the persistence of BB-MP in the environment has been found to be comparable to that of PB-plastics.

Our study also aimed to assess the sensitivity of two different *D. magna* clones. Despite this study being limited to only two separate clones, the results confirm that clonal differences play a significant role in determining the outcome of exposure studies. While MP-induced mortality did not differ between clones in our study, we found significant differences in sublethal responses. Accordingly, the genetic variability among different clones of *Daphnia* can influence the outcomes of toxicity studies, including those on MP, and should be investigated using molecular approaches in future investigations delving into this topic. Therefore, differences in clonal sensitivities should be considered when comparing the results of MP toxicity studies and understanding the impacts of MP in nature.

Another important aspect to consider in *D. magna* exposure studies is the inclusion of varying food levels to better reflect environmentally relevant conditions. While the present study was conducted under reduced food availability, future research should also examine particle exposure under different nutritional regimes. This would provide a more comprehensive understanding of how food availability modulates the biological responses to MP exposure.

CRediT authorship contribution statement

Christian Laforsch: Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Martin Wagner:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Martin G. J. Löder:** Writing – review & editing,

Validation, Supervision, Project administration, Investigation. **Seema Agarwal:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Dipannita Ghosh:** Writing – review & editing, Investigation. **Gabriel Olthof:** Writing – review & editing, Investigation, Conceptualization. **Marvin Kiene:** Writing – review & editing, Visualization, Formal analysis. **Michael Schwarzer:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Simona Mondellini:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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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. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2026.119934](https://doi.org/10.1016/j.ecoenv.2026.119934).

Data availability

Data will be made available on request.

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