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Reproductive toxicity of primary and secondary microplastics to three cladocerans during chronic exposure☆

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ABSTRACT

Microplastics (<5 mm) are distributed ubiquitously in natural environments. The majority of microplastics in aquatic environments are shown to have rough surfaces due to various weathering processes (secondary microplastics; SMP), while laboratory studies predominantly utilise pristine microplastics (primary microplastics; PMP). Here we present the results from a study comparing the chronic effects of pristine PMP and artificially weathered SMP to three different Cladoceran species (*Daphnia magna, Daphnia pulex, Ceriodaphnia dubia)*. We assessed the impact of PMP and SMP on reproductive output using various measured parameters, including time of first brood, size of first brood, size of first three broods, cumulative number of neonates, total number of broods and terminal length of test animals. Our results show that reproductive output of all species declined in a dose-dependent manner. The No Observed Effect Concentration (NOEC) was less than the lowest tested concentration (10^2 p/mL) for at least one measured endpoint for all species and both PMP and SMP. Further, it was inferred that species sensitivity varied inversely with body size for most endpoints, resulting in *C. dubia* being the most sensitive species; and *D. magna* being the least sensitive species under study. In addition, PMP appeared to have greater toxic potential as compared to SMP. This study is the first to directly compare the chronic toxicity of both pristine and weathered microplastic particles on three freshwater toxicological model organisms. Our results indicate that sensitivity in reproduction and growth to microplastics may differ between species and type of microplastic exposed; highlighting the importance of using multiple species and structural types of particles.

1. Introduction

Accumulation of small pieces of plastic (<5 mm), also known as microplastics, in aquatic habitats including marine (Browne et al., 2007; Galloway & Lewis, 2017), freshwater (Eriksen et al., 2013; Lechner et al., 2014; Peng et al., 2018; Su et al., 2016) and estuarine (Sruthy and Ramasamy, 2017) environments has received increasing attention in the recent years and concerns are arising regarding their potential adverse effects (e.g., Thompson et al., 2004; Eerkes-Medrano et al., 2015). In light of these discoveries, plastic pollution has been declared to be one of the most critical environmental issues of our time by the United Nations Environment Programme (UNEP, 2016).

Microplastics can be classified as primary or secondary microplastics based on whether they are manufactured to be of micron size or derived from fragmentation of macroplastics (Wright et al., 2013). Primary microplastics (PMP) are purposefully produced for commercial applications like personal care products (Gregory, 1996; Zitko & Hanlon, 1991). By contrast, secondary microplastics (SMP) are produced by degrading agents such as wave action, temperature changes and UV-B radiation in the environment (Andrady, 2011; Browne et al., 2007). The small sizes comparable to natural food particles coupled with the ubiquitous presence of microplastics, suggests the increased likelihood of ingestion by aquatic organisms (Browne et al., 2007; Steer et al., 2017). A range of laboratory and field studies have demonstrated the ability of various vertebrate and invertebrate taxa to ingest microplastics, including mussels and bivalves (Van Cauwenberghe et al., 2013, 2015); crustaceans (Murray and Cowie, 2011; Setälä et al., 2014);

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fish (Lusher et al., 2013; Neves et al., 2015); birds (Zhao et al., 2016; Holland et al., 2016) as well as marine mammals (Eriksson et al., 2013). Adverse impacts have also been documented in various organisms following uptake, such as teratogenicity (Nobre et al., 2015), inflammation (Lu et al., 2016), reduced energy reserves (Wright et al., 2013) as well as reduced feeding (Bergami et al., 2016).

Cladocerans are zooplanktonic freshwater branchiopods that form an ecologically important class of organisms. They constitute over 620 different species and generally fall in the size ranges of 0.2–18 mm (Forró et al., 2008). They represent a significant proportion of biomass in freshwater ecosystems (Culver et al., 1985) and play a vital role in energy transfer to higher trophic levels within aquatic food webs (Dodson & Frey, 2001). They undergo asexual reproduction to produce clutches of parthenogenetic eggs resulting in a rapid increase of population sizes (Ebert, 2005). Due to their short lifespans, rapid mode of reproduction, and ecological significance they are excellent model organisms to assess sub-lethal effects, such as depreciation in reproductive output during exposure to pollutants (OECD, 2012; USEPA, 2002).

Exposure of freshwater zooplanktonic species to microplastics may have adverse effects. For example, chronic exposure of the freshwater amphipod *Hyalella azteca* to (10 μm) polyethylene particles negatively impacted growth and reproduction (Au et al., 2015). Similarly, acute and chronic exposure of *Ceriodaphnia dubia* to polyester fibers resulted in significant effects on survival and reproduction (Ziajahromi et al., 2017). Studies on *Daphnia magna* have reported several findings such as altered food uptake rates during exposure to (100 nm) polystyrene microplastics (Rist et al., 2016) and immobilization, which increased with increasing exposure concentration and period, to 1 μm polyethylene particles (Rehse et al., 2016).

However, most studies assessing the toxicity of microplastics use a single-sized spherical model PMP (Phuong et al., 2016) for exposure experiments, although SMP are reported to have higher abundance in natural environments (Connors et al., 2017; Potthoff et al., 2017). In a recent study, Ogonowski et al. (2016) compared the toxicity of PMP and artificially weathered SMP on processes like feeding, growth as well as reproduction, during chronic exposure to *D. magna* and found that while PMP had limited impacts, exposure to SMP resulted in reduced reproductive output. Further, in an expansive study, we compared the sensitivity of three different Cladocerans during acute exposure to different types of microplastics in combination with thermal stress (Jaikumar et al., 2018), finding species-dependent sensitivity. However, such cross-species comparison of species sensitivity to differently shaped microplastics during chronic exposure has not been performed as of yet. Chronic toxicity assays are often more sensitive than acute assays and also more representative for ecotoxicological risk assessments (Newman & Dixon, 1996).

The goal of the current study was to increase our understanding on the chronic reproductive toxicity of both pristine (PMP) and artificially weathered (SMP) microplastics on three different freshwater Cladoceran species of different body sizes. To this end, we compared the reproductive toxicity of PMP and SMP on *Daphnia magna, Daphnia pulex,* and *Ceriodaphnia dubia*. Endpoints indicating the quality of reproductive output such as size of first brood, interval between broods, total number of neonates and total number of broods were assessed and compared between the species. We hypothesized that reproductive sensitivity is species-specific (influenced by body size of species) and dependent on the type of microplastic exposed.

2. Materials and methods

2.1. Test species and holding conditions

The three species used in this research have different sizes but similar life history traits: *Daphnia magna* (2–5 mm), *Daphnia pulex* (2–3 mm), and *Ceriodaphnia dubia* (<1.4 mm) (Clare, 2002; Balcer et al., 1984). *D. magna* and *D. pulex* reach sexual maturity, and produce the first parthenogenetic brood approximately around 7 days post-hatch, as opposed to four days in the case of *C. dubia* (OECD, 2012; USEPA, 2002).

Stock cultures of D. magna and *D. pulex* were kept at Leiden University and maintained as per OECD protocol 211 (OECD, 2012). Parent populations were maintained in 5-L aerated glass aquaria containing 4 L of Elendt M4 medium (pH of 7.0 ± 0.5; 22 ± 1 °C; 16:8 h light-dark cycle). Organisms were provided with a diet of *Pseudokirchneriella subcapitata* (10^4 cells/organism/day). Parent cultures were restarted once a month and sensitivity of the culture was tested once every 6 months as per OECD guidelines using Potassium dichromate (K_2CrO_7).

Stock cultures of *C. dubia* were maintained according to USEPA guidelines (USEPA, 2002). Parent populations were maintained in 3-L oxygenated glass tanks with 2 L of Elendt M4 (pH of 7.0 ± 0.5; 26 ± 1 °C; 16:8 h light-dark cycle). Organisms were provided with *P. subcapitata* and a diet of yeast, trout chow, and cerophyll extracts (YCT). Parent cultures were restarted once every 10–12 days to retain optimal vitality.

2.2. Preparation of microplastics

Spherical fluorescent primary microplastics (PMP; 1–5 μm, 1.30 g/cm^3; Cospheric LLC, Goleta, USA) were suspended in Elendt M4 medium by mixing followed by vortexing for 10 s. A stock solution (10^8 p/mL) was prepared and diluted to make the exposure concentrations used in the current experiment.

Secondary microplastics (SMP) were prepared following the protocol developed by Ogonowski et al. (2016). Briefly, polyethylene microspheres (850–1000 μm, 0.96 g/cm^3; Cospheric LLC, Goleta, USA) were ground (Retsch CryoMill; Retsch, Dusseldorf, Germany), and subsequently sieved to yield irregular and coarse particles, as a model for SMP. The size range of these particles was 1–10 μm (validated using TEM; see Jaikumar et al., 2018). Next, the particles were suspended using the surfactant Tween 80 (Sigma Aldrich), followed by serial centrifugation with Milli-Q water to remove the surfactant (Jaikumar et al., 2018). Finally, stock suspensions were prepared using Elendt M4 medium (10^7 p/mL), and further desired exposure concentrations were achieved through serial dilution. The concentrations of PMP and SMP were confirmed using a hemocytometer.

2.3. Chronic toxicity test

Prior to all experiments, individuals from all three test species were briefly exposed to both types of microplastic and observed under the microscope, to confirm the uptake and ingestion of both MP types by all species. A 21-d reproductive test was performed for *D. magna* and *D. pulex* in accordance with OECD guideline (OECD, 2012) at 22 ± 1 °C. Neonates (<24 h old) were held in 50-mL glass flasks containing 30 mL of Elendt M4 medium and exposed to control, 10^2, 10^3, 10^4 and 10^5 p/mL of both primary (PMP) and secondary (SMP) microplastics (one individual/beaker, 12 replicates/treatment and 24 replicates for controls). Medium was changed 3 times per week and water quality parameters like

dissolved oxygen and conductivity were ensured to be within the desired range prior to medium change. The individuals were fed with *P. subcapitata* algae at a dose of 1.5×10^5 cells/organism/day for the first week, and 3×10^5 cells/organism/day for the second and third weeks. A 16-8 h light-dark cycle and pH of 7 ± 1 were maintained. Throughout the experiment, the age of females at first brood (days), size of first brood, number of broods, size of first three broods, cumulative number of neonates, adult size at the end of 21 days (mm) were assessed. In all cases, overall control mortality was less than 20% and the average cumulative number of neonates produced per control was ≥60.

A 7-d reproductive test was conducted for *Ceriodaphnia dubia* in accordance with USEPA protocol (USEPA, 2002) at 26 ± 1 °C. As a minor modification to the protocol, neonates (<24 h old) were held in 15 mL of Elendt M4 medium (in place of Milli-Q water), and exposed to the same concentrations of both PMP and SMP as the other two species (one individual/beaker, 12 replicates/treatment and 24 replicates for controls). Medium was changed every day and the individuals were fed daily with a mixture of *P. subcapitata* and YCT (Yeast, Cerophyll, and Trout chow extract) in doses recommended by the USEPA protocol. A 16-8 h light-dark cycle and pH of 7 ± 1 were maintained. Throughout the experiment, the same endpoints were assessed as listed above for the other two species. Likewise, control mortality was less than 20% and the average cumulative number of neonates produced per control was ≥15.

2.4. Data analysis

To assess the effect of each type of microplastic (PMP or SMP) on endpoints measured for every species, one-way Analysis of Variance (ANOVA) tests were conducted. Prior to analysis, assumptions of normality and homoscedasticity were tested using a Bartlett's statistic test and Brown-Forsythe test, respectively. If significant differences were detected, Bonferroni post-hoc tests were employed for multiple comparisons between different treatments. Significance was set at $p \leq 0.05$ and all data reported as mean ± SEM. Based on these results, Lowest Observed Effect Concentration (LOEC) and No Observed Effect Concentration (NOEC) were computed for each species. All tests were carried out using GraphPad Prism (version 7.0c, GraphPad Software, San Diego, CA, USA). All test statistics (degrees of freedom, p-values, and F-values) are presented in Table S1.

3. Results

3.1. Effects on time of first brood

Exposure to PMP did not affect the age of individual at which first brood was produced significantly in any of the three species studied (Table 1). However, there was a significant effect of SMP on day of first brood for *D. pulex* at 10^2 p/mL and *C. dubia* at 10^5p/mL, although this effect was not observed at other exposure concentrations (Table 1).

3.2. Effects on size of first brood

PMP significantly influenced the size of first brood for all three species, at the concentrations of 10^3 p/mL for *D. magna*, 10^5 p/mL for *D. pulex* and 10^5 p/mL for *C. dubia,* respectively (Fig. 1). At the highest exposure concentration, PMP resulted in a 42%, 37% and

Table 1
Summary of chronic endpoints of *Daphnia magna*, *Daphnia pulex* and *Ceriodaphnia dubia* exposed to primary (PMP) and secondary (SMP) during 21 d and 7 d chronic reproductive tests. Values are means ± Standard Error of Mean (SEM) for all measured endpoints (n = 12).

Species	Type of MP	Concentration (particles mL-1)	Time of first brood (days)	First three broods (# of neonates)	Number of broods	Terminal Length (mm)
D. magna	PMP	Control	8.09 ± 0.20	39.65 ± 1.23	5.17 ± 0.10	3.73 ± 0.02
		10^2	8.17 ± 0.24	32.67 ± 1.79*	5.25 ± 0.13	3.69 ± 0.04
		10^3	8.17 ± 0.24	33.58 ± 1.39	5.09 ± 0.09	3.72 ± 0.03
		10^4	8.75 ± 0.13	31.17 ± 2.10**	5.00 ± 0.00	3.62 ± 0.04*
		10^5	8.73 ± 0.14	23.08 ± 2.98****	4.81 ± 0.12	3.55 ± 0.03
	SMP	Control	8.09 ± 0.20	39.65 ± 1.23	5.17 ± 0.10	3.73 ± 0.02
		10^2	8.58 ± 0.31	31.58 ± 1.22***	5.00 ± 0.12	3.74 ± 0.02
		10^3	8.08 ± 0.23	30.25 ± 1.30****	5.09 ± 0.16	3.71 ± 0.05
		10^4	7.91 ± 0.25	30.00 ± 1.77****	5.18 ± 0.12	3.78 ± 0.04
		10^5	8.25 ± 0.13	29.08 ± 1.40****	5.00 ± 0.12	3.72 ± 0.02
D. pulex	PMP	Control	7.10 ± 0.21	30.43 ± 1.87	6.75 ± 0.09	2.58 ± 0.03
		10^2	7.92 ± 0.62	21.25 ± 2.93*	6.09 ± 0.37	2.53 ± 0.04
		10^3	7.27 ± 0.27	22.67 ± 2.75	6.27 ± 0.14	2.56 ± 0.02
		10^4	6.64 ± 0.65	19.50 ± 2.64**	6.10 ± 0.18	2.50 ± 0.05
		10^5	8.00 ± 0.24	17.67 ± 1.76***	5.67 ± 0.14***	2.51 ± 0.02
	SMP	Control	7.10 ± 0.20	30.43 ± 1.87	6.75 ± 0.09	2.58 ± 0.03
		10^2	8.00 ± 0.27*	20.58 ± 1.92**	6.42 ± 0.15	2.58 ± 0.02
		10^3	7.70 ± 0.15	16.25 ± 2.32****	6.30 ± 0.15	2.50 ± 0.05
		10^4	7.40 ± 0.16		6.60 ± 0.16	2.58 ± 0.02
		10^5	7.27 ± 0.24	19.00 ± 3.11**	6.73 ± 0.14	2.54 ± 0.02
C. dubia	PMP	Control	4.00 ± 0.00	17.05 ± 0.59	3.00 ± 0.00	0.82 ± 0.01
		10^2	4.27 ± 0.14	12.73 ± 1.00**	2.63 ± 0.15	0.84 ± 0.02
		10^3	4.20 ± 0.13	12.10 ± 0.95***	2.70 ± 0.15	0.80 ± 0.01
		10^4	4.11 ± 0.11	12.89 ± 1.19**	2.89 ± 0.11	0.83 ± 0.02
		10^5	4.27 ± 0.14	8.10 ± 1.01****	2.55 ± 0.20*	0.73 ± 0.02***
	SMP	Control	4.00 ± 0.00	17.05 ± 0.59	3.00 ± 0.00	0.82 ± 0.01
		10^2	4.11 ± 0.11	14.22 ± 0.92	3.00 ± 0.00	0.81 ± 0.01
		10^3	4.09 ± 0.09	13.64 ± 0.95**	2.90 ± 0.09	0.80 ± 0.01
		10^4	4.00 ± 0.00	12.91 ± 0.60***	3.00 ± 0.00	0.80 ± 0.01
		10^5	4.30 ± 0.15*	10.70 ± 0.96****	2.80 ± 0.13*	0.82 ± 0.02

Significant differences from control: *p value ≤ 0.05, **p value ≤ 0.01, ***p value ≤ 0.001, ****p value ≤ 0.0001.

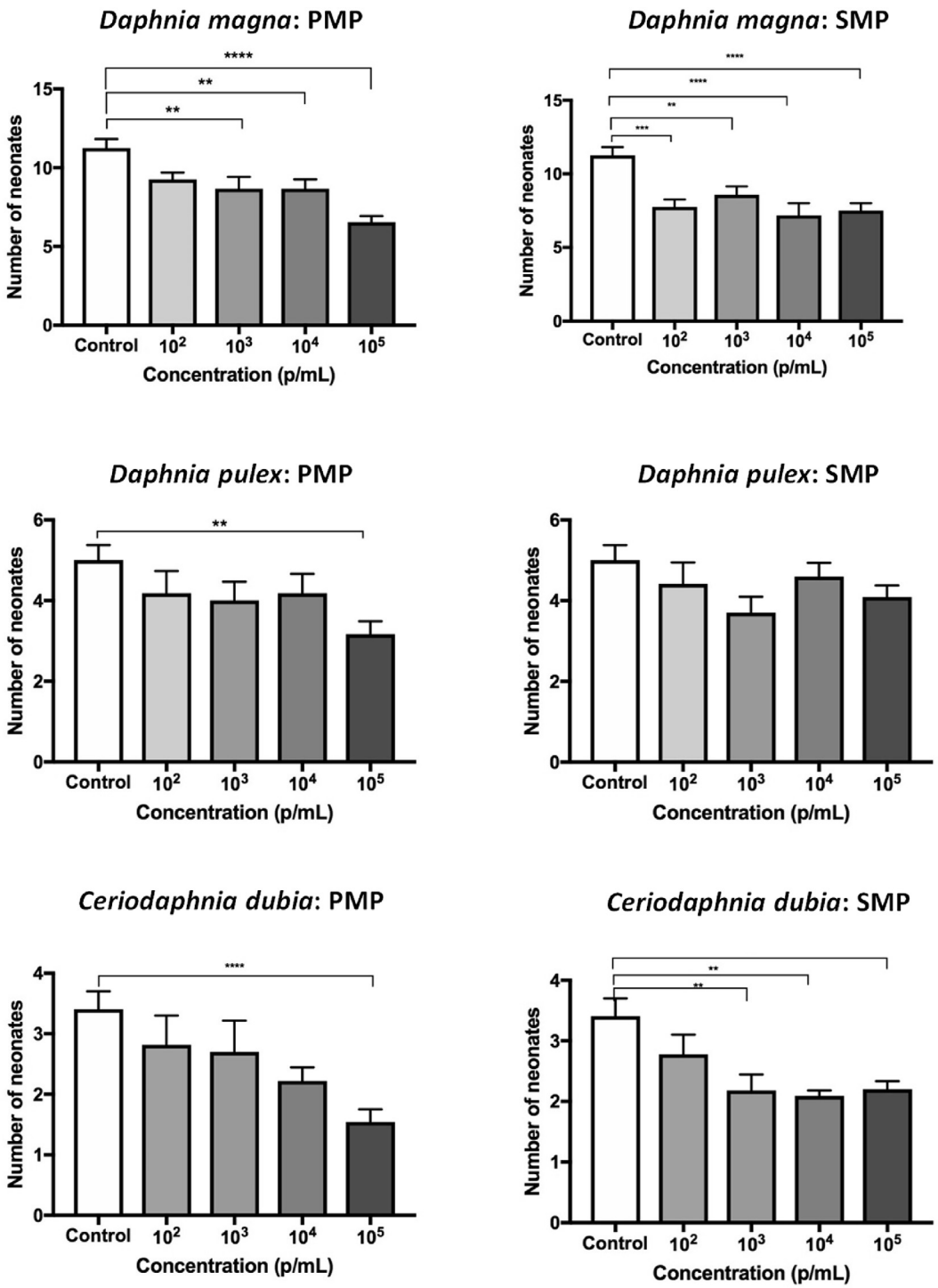

Fig. 1. Reduction in size of first brood during exposure of *Daphnia magna* and *Daphnia pulex* for 21 d and *Ceriodaphnia dubia* for 7 d to primary (PMP) and weathered (SMP) microplastics in chronic tests. Bars indicate means ± SEM (n = 12). Concentrations in particles/mL are shown on the x-axis, mean sizes of first broods produced are shown on the y-axis.

55% approximate reduction in the size of first brood for *D. magna*, *D. pulex,* and *C. dubia,* respectively.

SMP affected the size of first brood for *D. magna* even at the lowest exposure concentration of 10^2 p/mL and *C. dubia* at 10^3p/mL but had no effects on *D. pulex* (Fig. 1). At the highest exposure concentration, exposure to SMP resulted in an approximate reduction of 33%, 18%, and 35% in size of first brood for *D. magna*, *D. pulex,* and *C. dubia* respectively.

3.3. Effect on size of first three broods

There was an adverse effect of PMP exposure on the size of first three broods of all species, observed at the lowest concentration of 10^2 p/mL for *D. magna* as well as *D. pulex*, and *C. dubia*. Further, there was an approximate decrease of 42% for *D. magna*, 42% for *D. pulex,* and over 52% for *C. dubia,* in the size of first three broods, between control and highest exposure concentration (10^5 p/mL).

A similar adverse effect was also observed due to SMP at the lowest exposure concentration of 10^2 p/mL on *D. magna* and *D. pulex*, and at 10^3 p/mL for *C. dubia*. The resulting decreases in the size of first three broods as compared between control and highest exposure concentrations were approximately 27% for *D. magna*; 32% for *D. pulex* and 37% for *C. dubia*.

3.4. Effect on total number of broods

The total number of broods produced at the end of the reproductive assay was affected by PMP, albeit only at the highest exposure concentration of 10^5 p/mL (which was higher than the number of algal cells supplied as food source) for both *D. pulex* and *C. dubia*. Conversely *D. magna* was not significantly affected. Exposure to SMP exerted significant effects on total number of broods produced during the entire test period only for the smallest species *C. dubia* at the highest exposure concentration (Table 1).

3.5. Effect on cumulative number of neonates

Exposure to PMP had an adverse effect on cumulative number of neonates produced by all three species (Fig. 2). Significant effects were observed at 10^3 p/mL for *D. magna* but at the lowest concentration of 10^2 p/mL for *D. pulex* (Fig. 2). For *C. dubia* this endpoint is the same as size of first three broods, as only three broods were produced in the total duration of the reproductive assay. Further, there was approximately 20% decline for *D. magna* and 46% decline for *D. pulex* in the cumulative number of neonates produced, when comparing controls to the highest exposure concentrations.

Exposure to SMP had a similar negative effect on cumulative number of neonates for *D. magna* and *D. pulex* already at the lowest exposure concentration of 10^2 p/mL (Fig. 2). As a result, there was an approximate decline of 23% for *D. magna* and 34% decline for *D. pulex* in cumulative reproductive output as compared between control and highest exposure groups (Fig. 2).

3.6. Effect on terminal length

PMP also had an effect on growth of organisms as quantified by the terminal length measured at the end of reproductive assay, for *D. magna* at 10^4 p/mL and *C. dubia* at 10^5p/mL (Table 1). The resulting decrease in size of organisms, obtained by comparison of controls with highest exposure concentrations, was approximately 3% and 11% for *D. magna* and *C. dubia* respectively. However, SMP did not affect terminal length significantly for any of the species under study (Table 1). For ease of comparison, the NOEC and LOEC values of all endpoints are summarized in Table 2.

4. Discussion

Our results provide key insights into the chronic effects of both primary and artificially weathered secondary microplastics to three different Cladoceran species commonly used in ecotoxicological risk assessments. Chronic exposure to microplastics impaired reproductive output by influencing brood sizes, and different species indicated different sensitivities to the microplastics studied.

The sensitivity of different species can vary within several orders of magnitudes and is therefore of relevance for ecological risk assessment. When comparing cumulative number of neonates (indicating effect on net reproductive output) produced during exposure to highest concentration with the control, *C. dubia* was the most adversely affected and therefore the most sensitive, followed by *D. pulex* and *D. magna*, which were comparatively less sensitive at the highest exposure levels. This variation in species sensitivity may be inversely correlated with their body size (*D. magna: D. pulex: C. dubia* – 4.5:3:1 approximately) and therefore body volume. This is in line with previous studies comparing chemical toxicity of species with different body sizes, reporting the inverse correlation of sensitivity with body volume to copper nanoparticles, zinc, and microplastics (Song et al., 2015; Vesela and Vijverberg, 2007; Jaikumar et al., 2018). The metabolic rates of smaller sized species are higher than that of larger species (Vesela and Vijverberg, 2007). Since metabolic rate has a positive effect on uptake rates, it is possible that the smaller species accumulate greater amounts of microplastics per unit body mass, causing greater toxicity.

We previously assessed the acute toxicity of PMP and SMP on the same three species (Jaikumar et al., 2018) and reported that at 18 °C, species under study showed a similar acute sensitivity distribution as demonstrated in the present study (*C. dubia* > *D. magna* ≥ *D. pulex*). However, species sensitivity distributions may depend on the endpoint assessed. Some of the reproductive endpoints assessed in this study (e.g. size of first brood in SMP exposed Cladocerans; Fig. 1, Table 2) bring out *D. magna* as the most sensitive species, while endpoints such as size of first three broods (Table 2) show equal sensitivity across the different species. Supporting this, a study comparing species sensitivity during chronic exposure found that *D. magna* was more sensitive than *C. dubia* to silver nitrate (Naddy et al., 2007). These results reiterate that multiple endpoints and species comparisons are necessary to evaluate the environmental risk of different toxicants.

In comparison to SMP, PMP appeared to have higher levels of impact on reproductive output of all three species. Furthermore, effects on growth parameter (terminal length) were only caused by PMP on *D. magna* and *C. dubia*. Differences between PMP and SMP have been previously described in acute studies with three Cladocerans (Jaikumar et al., 2018) and a chronic study on *D. magna* (Ognwski et al., 2016). Likewise, a study of chronic toxicity of polyethylene microplastic particles of different shapes (beads and fibres) on *Ceriodaphnia dubia* reported differences in toxic potential of variably shaped particles (fibres > beads; Ziajahromi et al., 2017). This may be interpreted in light of the widely applicable Dynamic Energy Budget framework (Kooijman, 2001), which theorizes that the metabolic energy reserve derived from food is primarily used for the major functions of somatic and structural maintenance of organism, growth and maturation, and reproduction. Such consideration may be especially important when comparing across species, as metabolic rate and therefore, energy budgets vary with body size (Nisbet et al., 2000). As PMP appeared to have stronger effects on energy budgets allocated for growth as well as reproduction in comparison to SMP, they may be inferred to impact the overall metabolic energy reserve more detrimentally. However, cross species comparison is limited due to different feeding regimen. To confirm the energy budget hypothesis, more extensive studies with measurements of growth at multiple points throughout the duration of study and equal amounts of carbon fed for all species are necessary, as well as the differences between food regimes between the OECD protocol used for *D. magna* and *pulex* (*P. subcapitata*) and the USEPA protocol used for *C. dubia* (mixture of *P. subcapitata* and YCT).

Microplastics can also result in reduced feeding rates in organisms including *D. magna* (Rist et al., 2017; Ogonowski et al., 2016) which can impair the energy budget. The body size (length of carapax) and the mesh size of the filtering apparatus are defining the size range of particles ingested (Burns, 1968) allowing *D. magna* to ingest particles between 200 nm and 90 μm (Burns, 1968; Rist et al., 2017) and *C. dubia* to ingest particles with sizes up to 25 μm (Burns, 1968). Thus, the particle size tested in this study is in the feeding range of all Cladocerans tested here. However, it remains to be investigated whether the ingestion rate of early developmental

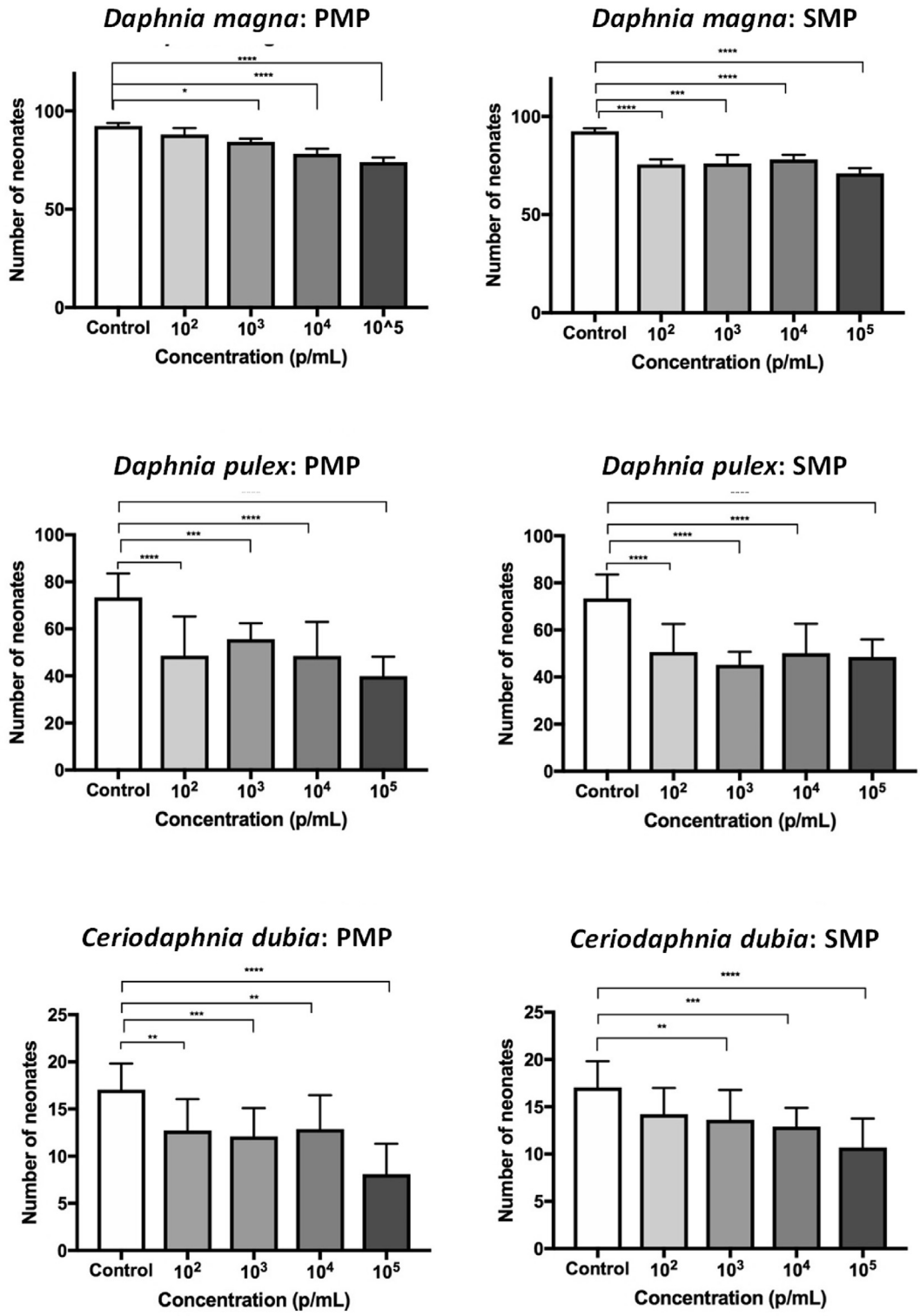

Fig. 2. Reduction in cumulative number of neonates produced during exposure of *Daphnia magna* and *Daphnia pulex* for 21 d and *Ceriodaphnia dubia* for 7 d to primary (PMP) and weathered (SMP) microplastics in chronic assays. Bars indicate means ± SEM (n = 12). Concentrations in particles/mL are shown on the x-axis, mean cumulative number of neonates produced are shown on the y-axis.

stages is equal to adult stages. A study on several freshwater invertebrates (including *D. magna*) found that the amount of ingested particles, as well as the maximum ingestible size of particles changes with different developmental stages (Scherer et al., 2017). Further research on the importance of development stage on microplastic effects is therefore required. In addition, a study on the freshwater cnidarian *Hydra attenuata* described a significant reduction in feeding rates following exposure to 400 μm

Table 2
No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) in particles/mL at which primary (PMP) and secondary microplastics (SMP) affected individual endpoints for *Daphnia magna*, *Daphnia pulex*, and *Ceriodaphnia dubia* during chronic test. ND: not determined.

Type of particle	Endpoint assessed	*D. magna*		*D. pulex*		*C. dubia*	
		LOEC	NOEC	LOEC	NOEC	LOEC	NOEC
PMP	Day of first brood	$>10^5$	ND	$>10^5$	ND	$>10^5$	ND
	Size of first brood	10^3	10^2	10^5	10^4	10^5	10^4
	Total # of broods	$>10^5$	ND	10^2	$<10^2$	10^5	10^4
	Size of first 3 broods	10^2	$<10^2$	10^2	$<10^2$	10^2	$<10^2$
	Cumulative # of neonates	10^3	10^2	10^2	$<10^2$	10^2	$<10^2$
	Terminal length	10^4	10^3	$>10^5$	ND	10^5	10^4
SMP	Day of first brood	$>10^5$	ND	10^2	$<10^2$	10^5	10^4
	Size of first brood	10^2	$<10^2$	$>10^5$	ND	10^3	10^2
	Total # of broods	$>10^5$	ND	$>10^5$	ND	$>10^5$	ND
	Size of first 3 broods	10^2	$<10^2$	10^2	$<10^2$	10^2	$<10^2$
	Cumulative # of neonates	10^2	$<10^2$	10^2	ND	10^2	$<10^2$
	Terminal length	$>10^5$	ND	$>10^5$	ND	$>10^5$	ND

polyethylene flakes (0.08 g/mL) for 60 min (Murphy & Quinn, 2018). This may reduce the energy intake; and the subsequent energy budget available for growth and reproduction as well as regulatory functions such as somatic maintenance (Kooijman, 2001). This is evidenced by the reduction in net reproductive output (cumulative number of neonates) and terminal body length of test organisms exposed to microplastics in the current study. In support, a study on reproductive effects of nanopolystyrene on *D. magna* also described lower growth, altered reproduction and severe physical malformations in neonates (Besseling et al., 2014). It is also hypothesized that microplastic aggregates cause internal abrasions or mechanical damage following ingestion (Ogonowski et al., 2016). As microplastics have been shown to cause a diverse array of effects and symptoms on exposed organisms, further investigations of mechanisms inducing toxicity are warranted.

In our study, we used PMP and SMP, which were composed of different polymers. However, it is unlikely that this impacted the results, as previous studies have confirmed that leachates of plastic additives do not elicit effects even at much higher concentrations to *D. magna* (Lithner et al., 2012). The particles also belonged to slightly different size ranges, with the PMP being generally smaller than SMP. Smaller sizes of the PMP may have been an attribute contributing to their higher potential toxicity. In addition, the particles also behaved differently in suspension as a function of their different densities. The PMP sank whereas the SMP was neutrally buoyant, which could have influenced their bioavailability for uptake. However, as Cladocerans are filter feeders that feed from the bottom as well as the water column, this effect may be minimal. Furthermore, as environmental microplastics are diverse in shape, composition, type and size, the present results suggest that eco-toxicological risk assessments of microplastics must use particles of different chemical compositions as well as physical shapes for exposure.

Importantly, the concentrations of exposure used in the present study are higher than those reported in the environment, highlighting that the risk of microplastics is currently low. However, the lack of uniform sampling and quantification techniques, standard methods and units of measurement (Besley et al., 2017; Phuong et al., 2016), in combination with geophysical influences (e.g. wind, water) and geographical variation cause enormous variability in observed and reported abundance of environmental microplastics. Therefore, the relative abundance of environmental microplastics in sizes comparable to those used in the present study are not well understood (Huvet et al., 2016; Lenz et al., 2016). It should also be noted that environmental concentrations of plastics and microplastics are likely to increase in the future because of the annual increase in plastic production and inability of plastics to undergo biological degradation (Eerkes-Medrano et al., 2015). This is in line with a recent detailed review concluding that ecological risks of microplastics are currently rare, but highlighting that if emissions continue (scenario: business as usual) risks may become widespread (SAPEA, 2019). In addition, we previously demonstrated that in the presence of environmentally relevant stressors such as thermal stress in combination with microplastic exposure, the sensitivity of species increased drastically (Jaikumar et al., 2018). More studies are necessary to understand the interactions between microplastics and other environmentally relevant stressors such as temperature, pH, salinity as well as other hazardous compounds to fully understand the ecological risks of microplastics.

5. Conclusion

Our results show that reproductive output of all species declined during exposure to both PMP and SMP. The NOEC was less than the lowest tested concentration (10^2 p/mL) for at least one measured end point. Further, by analysing effect sizes of most important end points of growth and reproduction, it was inferred that for some endpoints species sensitivity varied inversely with body size, resulting in *C. dubia* being the most sensitive species; and *D. magna* being the least sensitive species under study. Further, PMP particles appeared to have greater toxic potential as compared to SMP. Our results indicate different sensitivities between species and type of microplastic exposed; and reiterate the need for toxicological risk assessments using multiple species and types of particles.

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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.2019.03.085.

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