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## Sublethal impacts of fragmented polyethylene nanoplastics on *Daphnia magna* following chronic exposure

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Polyethylene (PE) and fragment-shaped particles are among the most prevalent polymer types and morphologies in aquatic environments. However, chronic effects of fragmented PE nanoplastics (NPs) on aquatic organisms remain largely unexplored. We evaluated the chronic toxicity (27 days) of PE NP fragments in *Daphnia magna* at 0.2 mg C L<sup>-1</sup> food level. Although *D. magna* ingested NP fragments, no significant changes in mortality were observed. However, high concentrations (10 and 100 µg mL<sup>-1</sup>) of NP fragments statistically decreased the number of offspring and reduced their body length. In addition, the proportions of males and stillborn offspring were statistically increased at 10 and 100 µg mL<sup>-1</sup> of NP fragments without changing the body length of those offspring. The population growth rate of *D. magna* exposed to NP fragments at 1, 10, and 100 µg mL<sup>-1</sup> was statistically lower than the control. It should be noted that the concentrations tested, particularly 10 and 100 µg mL<sup>-1</sup>, substantially exceed currently reported environmental NP concentrations. These findings provide chronic toxicity data for PE NP fragments that may contribute to NP risk assessment frameworks.

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### Environmental significance

Nanoplastics represent an emerging environmental concern, as plastic debris continuously fragments into particles smaller than 1000 nm that can penetrate biological membranes and potentially cause adverse effects in aquatic organisms. While numerous studies have examined nanoplastic toxicity, few have assessed the chronic effects of polyethylene nanoplastic fragments, despite polyethylene and fragmented shapes being among the most prevalent polymer types and morphologies in aquatic environments. This study evaluated the chronic toxicity of polyethylene nanoplastic fragments to *Daphnia magna* and demonstrated significant sublethal effects, including reduced reproduction, altered offspring fitness, and decreased population growth rate at high concentrations, despite no lethal toxicity. These findings provide toxicity data for polyethylene nanoplastic fragments that can contribute to nanoplastic risk assessment, although the concentrations tested substantially exceed currently reported environmental levels.

## 1. Introduction

Plastic pollution remains a pressing environmental issue, with microplastics (MPs, <5000 µm) and nanoplastics (NPs, <1 µm) increasingly recognized as emerging contaminants of global concern.<sup>1</sup> While the ecological impacts of MPs have been extensively investigated across various organisms and exposure scenarios, knowledge regarding the toxicity of NPs remains limited.<sup>2</sup> Despite this limited understanding, the smaller size,

higher surface reactivity, and potential for tissue penetration of NPs introduce additional layers of ecological complexity that are only beginning to be understood.<sup>3</sup> Addressing these knowledge gaps is essential for a more complete assessment of plastic pollution impacts at the nanoscale.

Evidence from MP studies demonstrates that fragment-shaped particles induce more severe adverse effects, including immobilization, oxidative stress, impaired reproduction, and increased gut retention time, compared with their spherical counterparts.<sup>4-6</sup> These findings suggest that particle morphology plays a role in determining toxicity outcomes. Similarly, shape-dependent differences in toxicity may also occur at the nanoscale.<sup>2,7</sup> However, direct evidence for shape-dependent toxicity of NPs remains limited, and further investigation is warranted. NP toxicity research has predominantly focused on spherical particles, particularly polystyrene (PS) spheres,<sup>8,9</sup> due to their commercial availability and ease of synthesis. Polyethylene (PE), the most abundant polymer in

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aquatic environments,<sup>10–12</sup> predominantly exists in irregular shapes.<sup>12,13</sup> Ecological risks of NPs manifest at the organism level through mortality, reduced growth, and reproductive alteration in aquatic organisms.<sup>14–16</sup>

To address the limited data on PE NP fragment toxicity, the present study evaluated the chronic ecotoxicity of PE NP fragments in the freshwater crustacean *Daphnia magna*. We previously manufactured these PE NP fragments using a focused ultrasonic system that incorporated both physical (cavitation and collapse) and chemical (UV weathering) processes to simulate natural degradation.<sup>17</sup> *D. magna* was selected as the test organism due to its sensitivity to toxicants, wide distribution in the Northern Hemisphere freshwater, and short generation time. To identify chronic effects of PE NP fragments, *D. magna* was exposed to the NPs for 21 days across a concentration gradient (0.1–10 µg mL<sup>-1</sup>). Lethal (survival rate) and sublethal endpoints (number of offspring, time to first brood, developmental ratio, sex ratio, and body length) were evaluated. The results provide chronic toxicity data for PE NPs and contribute to the NP ecotoxicology literature, facilitating environmental risk assessments.

## 2. Materials and methods

### 2.1. PE NP fragments

PE NP fragments used in this study were manufactured following the methods described by Hwangbo *et al.*<sup>17</sup> Fluorescent green high-density PE beads (850–1000 µm; Cospheric LLC, CA, USA) were fragmented using a cylindrically focused ultrasonic system (400 kHz frequency and 150 W energy output) to nanosized plastics (means with a standard deviation of 85.14 ± 5.37 nm; range of 25–350 nm). The ultrasonic irradiation for 3 hours induced cavitation and sonoluminescence, including UV wavelengths, which resulted in surface oxidation and mechanical fragmentation of the PE beads. Unlike spherical NPs commonly used in toxicity studies, these fragments exhibited irregular and rock-like rounded shapes as confirmed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Fourier-transform infrared spectroscopy (FT-IR) analysis revealed that the fragmentation process introduced oxygen-containing functional groups, including OH and C–O bonds, on the particle surfaces. This indicated surface oxidation typical of environmentally weathered plastics. These physicochemical characteristics have been detailed in our previous study.<sup>17</sup>

### 2.2. Chronic toxicity tests of PE NP fragments

*D. magna* used were sourced from the National Institute of Environmental Research (NIER), Republic of Korea. The organisms were cultured in two 1 L glass beakers containing M4 medium (means with a standard deviation of pH 7.8 ± 0.1; hardness 250 mg CaCO<sub>3</sub> L<sup>-1</sup>). Adult females, synchronized in their reproductive cycles, were placed at a density of 20 individuals per beaker. The medium was renewed daily, and the daphnids were fed *Chlorella vulgaris* (2.0 mg carbon L<sup>-1</sup> day<sup>-1</sup>). Neonates from the third brood were collected for subsequent experiments.

The chronic toxicity test (27 days) was conducted using *D. magna* (<24 h old) at 20 °C under a 16 : 8 h light:dark cycle. One *D. magna* was added to a 50 mL glass beaker containing 50 mL of M4 medium, with ten replicates for each concentration of PE NP fragments [0.1, 1, 10, and 100 µg mL<sup>-1</sup>, including the control (M4 medium)]. With renewal of M4 medium every two days, *Chlorella vulgaris* was fed daily at 0.2 mg carbon L<sup>-1</sup> to minimize potential food-mediated interference with NP toxicity detection. Carbon content was calculated using a conversion factor for freshwater green algae<sup>18</sup> as follows:

$$\text{Carbon content} = 0.1204 \times V^{1.051} \text{ (mg carbon cell}^{-1}\text{)},$$

where  $V$  represents the cell volume (µm<sup>3</sup>). To prevent agglomeration of NP fragments and algae, the medium was mixed five times daily by gentle pipetting without disturbing the *D. magna*. Since the density of PE (0.99–1.01 g cm<sup>-3</sup>) used in this study is similar to that of water, this mixing procedure facilitated uniform distribution of particles throughout the water column and minimized surface accumulation. All *D. magna* were checked daily to evaluate survival, the number of offspring and eggs, and the time to first brood. Based on the number of females, males, and stillbirths, sex ratio and embryonic development were calculated as follows:

$$\text{Sex ratio} = \frac{B}{A + B} \times 100(\%),$$

$$\text{Embryonic development} = \frac{A + B}{A + B + C} \times 100(\%),$$

where  $A$ ,  $B$ , and  $C$  refer to the number of females, males, and stillbirths, respectively. The body lengths of the neonates were measured immediately after all offspring from each brood had been released, and adult body lengths were recorded at the end of the test. Images of the PE NP fragments in *D. magna* were analyzed using an inverted fluorescence microscope (EVOS, Thermo Fisher Scientific, Waltham, MA, USA). The body length of *D. magna* was measured from immediately above the eyespot to the base of the spine. Population growth rates were calculated using the Euler–Lotka equation:<sup>19</sup>

$$\sum_{x=1}^n l_x m_x e^{-rx} = 1,$$

where  $l_x$  represents the fraction of individual *D. magna* surviving until age  $x$ ,  $m_x$  represents the number of offspring per surviving female *D. magna* between the ages  $x$  and  $x + 1$ , and  $r$  represents the population growth rate. Finally, the effective concentrations causing 10 and 50% effects (EC<sub>10</sub> and EC<sub>50</sub>, respectively) on sublethal endpoints were calculated using nonlinear regression analyses in CETIS (v.1.8.7; Tidepool Scientific Software).

### 2.3. Statistical analyses

All statistical analyses were performed using SPSS version 25.0 (IBM Corp., Armonk, NY, USA). Daily survival rates were compared using the log-rank test with the Kaplan–Meier procedure. Statistical differences among the concentrations of PE NP fragments



were analyzed using one-way analysis of variance (ANOVA) followed by *post-hoc* Tukey's honestly significant difference test, and the Kruskal–Wallis test followed by the *post-hoc* Mann–Whitney test with Bonferroni correction for parametric and non-parametric data, respectively. Data were checked for normality of distribution using the Shapiro–Wilk test and homogeneity of variance using Levene's test. For all statistical tests, the significance level was set at  $\alpha = 0.05$ . Statistical differences were determined when  $p < \alpha$ . Data were described as the mean  $\pm$  standard deviation (SD). Figures are presented as box-and-whisker plots, displaying the interquartile range (IQR, the box), mean ( $\times$ symbol), median (horizontal bar within the box) and whiskers extending to 1.5 times the IQR. Outliers are shown as individual points.

### 3. Results

#### 3.1. Lethal toxicity of PE NP fragments

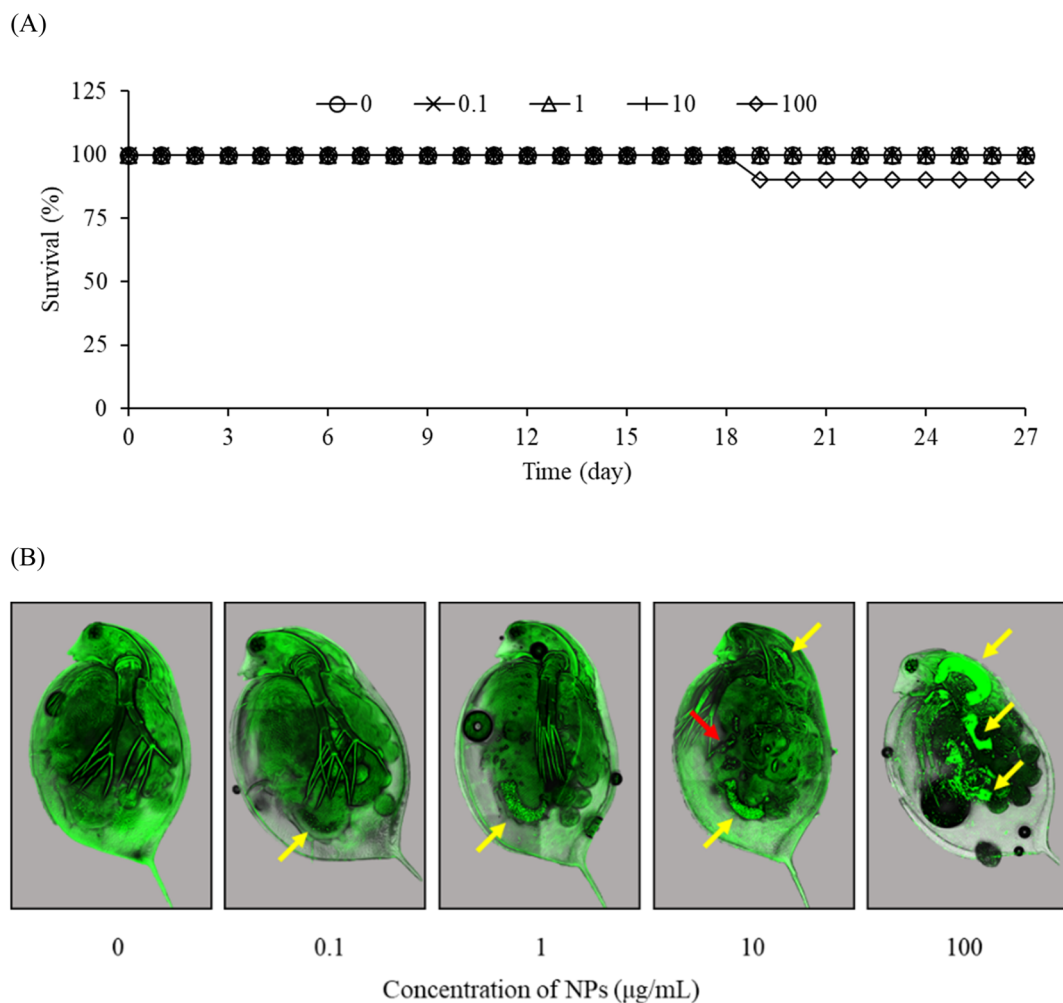
Survival of *D. magna* remained high across all PE NP fragment concentrations during the 27-day exposure, with only one

mortality observed at 100  $\mu\text{g mL}^{-1}$  on day 19 (Fig. 1A). No statistical differences ( $p > 0.05$ ) in survival were observed between treatments. Despite the ingestion of PE NP fragments by *D. magna* (Fig. 1B), the particles did not induce lethal toxicity.

#### 3.2. Sublethal toxicity of PE NP fragments

The PE NP fragments did not delay the time to first brood at any concentration (Fig. 2A) with  $\text{EC}_{10}$  and  $\text{EC}_{50}$  values exceeding 100  $\mu\text{g mL}^{-1}$  (Table 1). However, the number of offspring was the most sensitive endpoint, with  $\text{EC}_{10}$  and  $\text{EC}_{50}$  values of 1.32 and 4.98  $\mu\text{g mL}^{-1}$  (Table 1). However, the number of offspring and body length of adults statistically decreased ( $p < 0.05$ ) at higher concentrations (10 and 100  $\mu\text{g mL}^{-1}$ ) compared to the control (Fig. 2B and C).

High concentrations (10 and 100  $\mu\text{g mL}^{-1}$ ) of PE NP fragments statistically altered the sex ratio (Fig. 3A) and impaired embryonic development of offspring (Fig. 3B). For embryonic development,  $\text{EC}_{10}$  was 3.66  $\mu\text{g mL}^{-1}$ , while the  $\text{EC}_{50}$  exceeded 100  $\mu\text{g mL}^{-1}$  (Table 1). These concentrations statistically



**Fig. 1** (A) Daily survival rate and (B) images of *Daphnia magna* exposed to polyethylene (PE) nanoplastic (NP) fragments for 27 days. Daily survival curves were analyzed using the log-rank test with the Kaplan–Meier procedure. There were no statistical differences ( $p = 0.406$ ) between NP concentrations. Yellow and red arrows indicate the presence of PE NP fragments in the gut and on the thoracic limbs of *Daphnia magna*, respectively.





Fig. 2 (A) Time to first brood, (B) number of offspring, and (C) body length of *Daphnia magna* exposed to polyethylene (PE) nanoplastic (NP) fragments for 27 days. Data are presented as box-and-whisker plots ( $n = 10$ ). Different lowercase letters indicate statistical differences ( $p < 0.05$ ) between NP concentrations. Time to first brood was analyzed using the Kruskal–Wallis test, showing no statistical differences ( $p = 0.054$ ). Number of offspring and body length of adults were analyzed using one-way ANOVA ( $p < 0.001$  for both) followed by Tukey's *post-hoc* test. Box-and-whisker plots represent the interquartile range (the box), mean ( $\times$ symbol), median (horizontal bar within the box), and the minimum and maximum values (whiskers). Outliers are shown as individual points.

Table 1 The effective concentrations (ECs) for sublethal endpoints in *Daphnia magna* exposed to polyethylene nanoplastic fragments for 27 days. EC values and 95% confidence interval (CI) were calculated using a nonlinear regression procedure in CETIS

Endpoints	EC <sub>10</sub> (µg mL <sup>-1</sup> ) with 95% CI	EC <sub>50</sub> (µg mL <sup>-1</sup> ) with 95% CI
Time to first brood	>100	>100
Number of offspring per female	1.32 (1.10–1.51)	4.98 (4.65–5.40)
Embryonic development	3.66 (2.80–6.40)	>100





Fig. 3 (A) Sex ratio, (B) developmental ratio, and (C) body length of offspring from female *Daphnia magna* exposed to polyethylene (PE) nanoplastic (NP) fragments for 27 days. Data are presented as box-and-whisker plots ( $n = 10$ ). Different lowercase letters indicate statistical differences ( $p < 0.05$ ) between NP concentrations. Sex ratio and developmental ratio were analyzed using the Kruskal–Wallis test ( $p < 0.001$  for both) followed by the Mann–Whitney *post-hoc* test with Bonferroni correction. Body length of offspring was analyzed using one-way ANOVA ( $p = 0.038$ ) followed by Tukey's *post-hoc* test. Box-and-whisker plots represent the interquartile range (the box), mean ( $\times$ symbol), median (horizontal bar within the box), and the minimum and maximum values (whiskers). Outliers are shown as individual points.

increased ( $p < 0.05$ ) male offspring production to  $18.4 \pm 13.8$  and  $14.8 \pm 7.6\%$ , respectively, compared to the control ( $0.3 \pm 0.6\%$ ). Stillborn offspring production also statistically increased ( $p < 0.05$ ) to  $13.6 \pm 10.1\%$  and  $18.7 \pm 14.4\%$  at 10 and 100  $\mu\text{g mL}^{-1}$ , respectively, compared to the control ( $0.3 \pm 1.0\%$ ). Despite low embryonic development, well-developed offspring showed no statistical changes ( $p > 0.05$ ) in body length with any concentration of PE NP fragments (Fig. 3C). The population growth rate, which integrates the effects on the survival and reproductive outputs of female *D. magna*, was altered at most concentrations of NP fragments (Fig. 4).

## 4. Discussion

### 4.1. Lethal toxicity of PE NP fragments

The low mortality observed in our study may be attributed to the behavior of PE NP fragments in the water column. Although HDPE had a density ( $0.99\text{--}1.01 \text{ g cm}^{-3}$ ) close to that of water, visual observations revealed that agglomerated particles gathered at the water surface (Fig. S1). This surface accumulation potentially limited contact between particles and *D. magna* in the water column. Supporting our results, Coady *et al.*<sup>20</sup> reported no statistically significant mortality in *D. magna* exposed to ethylene acrylic acid copolymer ( $0.92 \text{ g cm}^{-3}$ ) NP beads



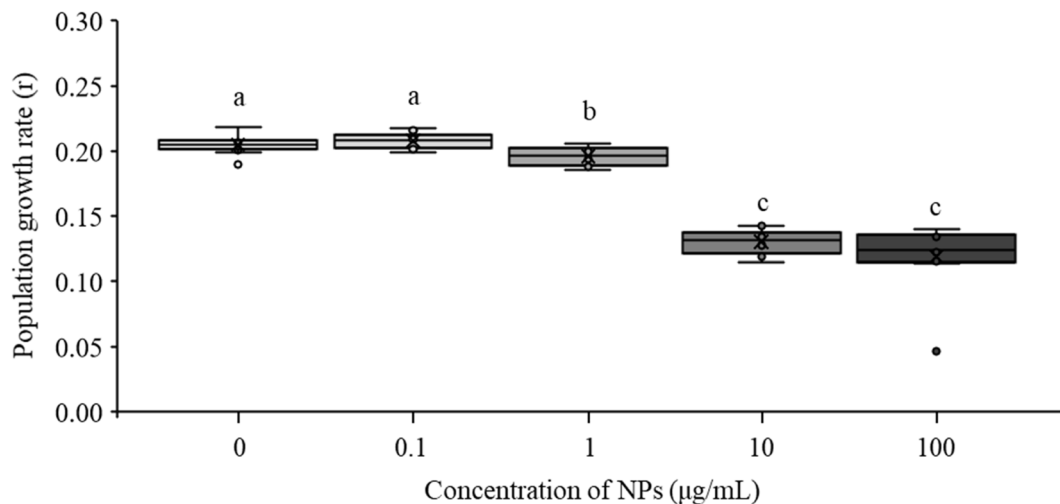


Fig. 4 Population growth rate of *Daphnia magna* exposed to polyethylene (PE) nanoplastic (NP) fragments for 27 days. Data are presented as box-and-whisker plots ( $n = 10$ ). Different lowercase letters indicate statistical differences ( $p < 0.05$ ) between NP concentrations. Population growth rate was analyzed using the Kruskal–Wallis test ( $p < 0.001$ ) followed by the Mann–Whitney *post-hoc* test with Bonferroni correction. Box-and-whisker plots represent the interquartile range (the box), mean ( $\times$  symbol), median (horizontal bar within the box), and the minimum and maximum values (whiskers). Outliers are shown as individual points.

(103 nm; 0.46 and 4.6  $\mu\text{g mL}^{-1}$ ) compared to the control for 21 days. In contrast, several studies have reported statistical mortality in *D. magna* exposed to PS (1.04–1.07  $\text{g cm}^{-3}$ ) NP beads.<sup>21–23</sup> These divergent outcomes in mortality may be attributed to polymer density differences. PS is denser than water and settles more readily in the water column, potentially increasing ingestion rates and lethal toxicity compared to PE.<sup>24</sup>

#### 4.2. Sublethal toxicity of PE NP fragments

Similar reproductive and growth impairments have been reported in daphnids exposed to PS NP beads for 21 days.<sup>16,25,26</sup> For instance, Liu *et al.*<sup>26</sup> observed that PS NP beads (75 nm) statistically decreased the number of offspring and body length of adult *Daphnia pulex* at low concentrations (0.1, 0.5, 1, and 2  $\mu\text{g mL}^{-1}$ ). In comparison, our study with PE NP fragments showed adverse effects only at relatively higher concentrations (10 and 100  $\mu\text{g mL}^{-1}$ ). This difference in effective concentrations may be attributed to multiple factors that distinguish these studies: (1) polymer density, with PS (1.05  $\text{g cm}^{-3}$ ) exhibiting better suspension than HDPE (0.99–1.01  $\text{g cm}^{-3}$ );<sup>24</sup> (2) particle size distribution, with monodisperse PS beads being more efficiently ingested than polydisperse PE fragments (25–350 nm);<sup>27</sup> and (3) species sensitivity, as *D. pulex* may be more sensitive than *D. magna*.<sup>28</sup> The mechanisms underlying these reproductive and growth impairments may involve oxidative stress responses.<sup>16,26</sup> Sanpradit *et al.*<sup>16</sup> demonstrated that reproductive capacity of *D. magna* was impaired by oxidative stress induced by PS NP bead exposure (50 nm).

Zhang *et al.*<sup>29</sup> demonstrated that NPs upregulated sex determining genes (DMRTA and DMRT1), statistically altering the sex ratio by increasing male offspring production in *D. pulex* exposed to PS NPs (75 nm; 1  $\mu\text{g mL}^{-1}$ ) for 21 days. Furthermore, Cui *et al.*<sup>30</sup> ascertained that *Daphnia galeata* exposed to PS NPs

(52  $\pm$  5 nm; 5  $\mu\text{g mL}^{-1}$ ) for 5 days stored significantly fewer and smaller lipid droplets in their bodies than the control, resulting in extremely high stillbirth rates (83  $\pm$  25%).

Two studies have reported reduced body size of offspring *D. magna* exposed to PS NP beads for 21 days, suggesting different reproductive strategies.<sup>16,25</sup> Sanpradit *et al.*<sup>16</sup> reported that NP exposure resulted in fewer and smaller offspring. In contrast, Besseling *et al.*<sup>25</sup> reported a trade-off between clutch size and offspring size, with a greater number of smaller offspring being released. In this study, PE NP fragment exposure at 10  $\mu\text{g mL}^{-1}$  resulted in a reduced number of offspring across all broods, indicating reproductive stress. Notably, the second brood exhibited fewer but longer offspring (Fig. S2), while other broods showed reduced numbers without changes in body length (data not shown). However, body length provides limited information on offspring quality, as it does not account for body width or mass. Therefore, body width or dry weight measurements would be needed to determine whether this second brood pattern represents a reproductive trade-off or stress-induced morphological alteration.

Except for 0.1  $\mu\text{g mL}^{-1}$ , all concentrations (1, 10, and 100  $\mu\text{g mL}^{-1}$ ) of PE NP fragments statistically decreased ( $p < 0.05$ ) the population growth rate of *D. magna* compared to the control. Previous studies have reported population growth rate reductions in daphnids exposed to PS NP beads for 21 days. Liu *et al.*<sup>14</sup> found statistical effects in *D. pulex* at 2  $\mu\text{g mL}^{-1}$  (71.18 nm) compared to the control. Lyu *et al.*<sup>22</sup> observed a statistically reduced population growth rate in *D. magna* at 1 and 2  $\mu\text{g mL}^{-1}$  (70 nm) at standard food levels (1.5  $\text{mg C L}^{-1}$ ) compared to the control, with similar effects at 2  $\mu\text{g mL}^{-1}$  under low food conditions (0.3  $\text{mg C L}^{-1}$ ).

To evaluate the environmental risk of PE NP fragments, we applied the GHS (UN Globally Harmonized System) classification criteria for long-term aquatic hazards.<sup>31</sup> Both EC<sub>10</sub> values for the



Table 2 Studies investigating the chronic toxicity of nanoplastics to daphnids<sup>a</sup>

NPs							
Shape	Polymer	Size (nm)	Conc. ( $\mu\text{g mL}^{-1}$ )	Species	Exposure duration (day)	Effects	References
Fragment	HDPE(f)	85.14 $\pm$ 5.37 (25–350)	0.1, 1, 10, 100	<i>D. magna</i>	27	Number of offspring/female: $\downarrow$ (10, 100) Body length of adult: $\downarrow$ (10, 100) Sex ratio: $\uparrow$ (10, 100) Embryonic development: $\downarrow$ (10, 100) Population growth rate: $\downarrow$ (1, 10, 100) Survival, time to 1st brood, number of brood, body length of offspring: no significant changes	This study
	HDPE	112 $\pm$ 4 (90–200)	0.53	<i>D. magna</i>	134	Survival: $\downarrow$ Number of offspring/female: no significant change	34
Bead	EAA	103	0.46 and 4.6	<i>D. magna</i>	21	Survival, number of offspring/female, body length of adult: no significant change	20
	PS-COOH(f)	20	0.1, 50	<i>D. magna</i>	21	Time to 1st brood: $\uparrow$ (50) Body length of adult: $\downarrow$ Number of offspring/female: no significant change	15
		200	50	<i>D. magna</i>	21	Body length of adult: $\downarrow$ Time to 1st brood: no significant change	
	PS(f)	100	0.1, 0.5, 1	<i>D. magna</i>	21	Number of offspring/female, time to 1st brood, number of brood, body length of adult: no significant changes	42
		94.5 $\pm$ 3.9	1.7, 3.5, 7.1, 14.3, 28.6	<i>D. magna</i>	21	Survival: $\downarrow$ (14.3, 28.6) Time to 1st brood: $\uparrow$ (14.3) Body length of adult: $\downarrow$ (14.3)	23
	PS	90.1 $\pm$ 4.9	1.6, 3.3, 6.6, 13.3, 26.6	<i>D. magna</i>	21	Survival: $\downarrow$ (26.6) Time to 1st brood: $\uparrow$ (13.3, 26.6) Body length of adult: $\downarrow$ (26.6)	
		70	1, 2	<i>D. magna</i>	27	Survival: $\downarrow$	22
		50	1, 2	<i>D. magna</i>	21	Number of offspring/female: $\downarrow$ Number of offspring/female: $\downarrow$ - Body length of adult: $\downarrow$ (2) Body length of offspring: $\downarrow$ (1st brood) Time to 1st brood: no significant change	16
		50	5	<i>D. magna</i>	21	Survival: $\downarrow$ Time to 1st brood: $\uparrow$ Number of brood: $\downarrow$ Number of offspring/female: no significant change	21
		72.84 $\pm$ 6.81	1.65, 3.31, 6.62, 13.24	<i>D. magna</i>	21	Survival, time to 1st brood, body length of adult: no significant changes	43
	71.18	0.1, 0.5, 1, 2	<i>D. pulex</i>	21	Number of offspring/20 females: $\downarrow$ (0.5, 2)	14	
	71.18	0.001	<i>D. pulex</i>	21		37	



Table 2 (Contd.)

NPs							
Shape	Polymer	Size (nm)	Conc. ( $\mu\text{g mL}^{-1}$ )	Species	Exposure duration (day)	Effects	References
		75	0.1, 0.5, 1, 2	<i>D. pulex</i>	21	Number of offspring/female, number of brood, body length of adult: no significant changes	
						Number of offspring/female: ↓	26
						Time to 1st brood: ↑	
						Number of brood: ↓	
						Body length of adult: ↓	
Unclear	PE	50 ± 10	0.00025	<i>D. magna</i>	21	Number of offspring/female: ↓ (5th brood)	35
	PP	53 ± 5	0.00022	<i>D. magna</i>	21	Survival: no significant change	
						Number of offspring/female: ↑ (5th brood)	
	PS	207 ± 12	0.13	<i>D. magna</i>	21	Survival: no significant change	
						Number of offspring/female: ↑ (4th brood), ↓ (5th brood)	
						Survival: no significant change	
						Number of offspring/female: ↑ (5th brood)	
	PVC	241 ± 28	0.17	<i>D. magna</i>	21	Survival: no significant change	
						Number of offspring/female: ↑ (5th brood)	
						Survival: no significant change	
	PS	75	1	<i>D. pulex</i>	21	Sex ratio: ↑	29

<sup>a</sup> HDPE: high density polyethylene; f: fluorescence; EAA: ethylene acrylic acid copolymer; PS: polystyrene; PE: polyethylene; PP: polypropylene; PVC: polyvinyl chloride.

number of offspring and embryonic development exceeded the threshold for Category Chronic 2 ( $\leq 1 \text{ mg L}^{-1}$ ), indicating that PE NP fragments do not meet the criteria for GHS chronic hazard classification (Category Chronic 1:  $\leq 0.1 \text{ mg L}^{-1}$ ; Category Chronic 2:  $\leq 1 \text{ mg L}^{-1}$ ). However, it is important to note several experimental considerations that may influence the interpretation of these results. In the present study, we did not follow OECD TG NO. 211 guidelines,<sup>32</sup> as food levels ( $0.2 \text{ mg C L}^{-1}$ ) were intentionally reduced to minimize potential interference with NP toxicity detection. However, this approach may have influenced the ability of *D. magna* to withstand potential stress from NP exposure. Lyu *et al.*<sup>22</sup> demonstrated that at low food levels ( $0.3 \text{ mg C L}^{-1}$ ), toxic effects of NPs on reproduction may be partially masked because organisms have already reached minimal reproductive output. Similarly, Sun *et al.*<sup>33</sup> showed that below  $0.3 \text{ mg C L}^{-1}$  food levels, the negative effects of ZnO nanoparticles on survival time and reproduction of *D. magna* were masked by food limitation. Under standard food conditions, *D. magna* may exhibit greater sensitivity to NP exposure, potentially yielding lower  $\text{EC}_{10}$  values.

#### 4.3. Implications and limitations

Numerous studies have assessed the physiological effects of NPs on *D. magna* (Table 2), focusing on PS beads. Few studies have investigated the chronic toxicity of PE NPs with fragmented shapes in daphnids. Ekvall *et al.*<sup>34</sup> reported that HDPE

( $0.97 \text{ g cm}^{-3}$ ) NP fragments ( $112 \pm 4 \text{ nm}$ ) statistically decreased survival of *D. magna* for 134 days. This study used commercial packaging material mixed with tap water and fragmented with a hand-held food blender, which was filtered using an 800 nm syringe filter. The filtrate contained all molecules and materials that could pass through the filter. Thus, the concentrations and additives of NPs were unspecified, indicating that the properties of NPs are toxic to *D. magna*. Additionally, Monikh *et al.*<sup>35</sup> investigated the reproductive toxicity of PE (50 nm;  $0.00025 \mu\text{g mL}^{-1}$ ), PP (50 nm;  $0.00022 \mu\text{g mL}^{-1}$ ), PS (200 and 600 nm;  $0.13$  and  $3.5 \mu\text{g mL}^{-1}$ , respectively), and PVC (200 nm;  $0.17 \mu\text{g mL}^{-1}$ ) NPs in *D. magna* for 21 days. All NPs statistically altered the number of offspring in the fourth and fifth broods of *D. magna*, whereas earlier broods remained largely unaffected. However, the authors focused on characterizing the changes in NPs caused by co-occurring benzo( $\alpha$ )pyrene and on toxicity to *Danio rerio*. While the NP morphology was not explicitly stated in the paper, microscopic images revealed fragmented shapes. Toxicological studies (Table 2) have used different NP materials, sometimes without providing specific information on their characteristics, which makes comparing the toxic effects of NPs across studies challenging. Sørensen *et al.*<sup>36</sup> suggested that test materials should incorporate environmental weathering processes and physicochemical characteristics to enhance environmental relevance.



Cross-study comparisons are further complicated by variability in experimental protocols among published NP toxicity studies. Although most NP toxicity studies listed in Table 2 report adherence to OECD TG NO. 211 guidelines,<sup>32</sup> several studies have modified the standard chronic test protocols by altering test vessel volume per organism,<sup>15,21,22</sup> conducting group rather than individual exposures,<sup>14,22</sup> or extending test duration.<sup>22,34</sup> Additionally, some studies did not follow standardized chronic toxicity protocols,<sup>14,26,34,37</sup> including the present study. These methodological differences should be considered when comparing toxicity results across studies.

The present study evaluated the chronic toxicity of PE NP fragments to the freshwater cladoceran *D. magna*, using materials representing one of the predominant polymer types and morphologies observed in natural aquatic environments.<sup>12</sup> Although no significant mortality was observed, this study revealed sublethal toxicities, including reduced reproduction and altered offspring sex ratio at high concentrations (10 and 100  $\mu\text{g mL}^{-1}$ ), indicating potential population-level impacts. The concentrations tested in this study require careful consideration regarding environmental relevance. Materić *et al.*<sup>10</sup> reported NP concentrations ranging from 24 to 1588  $\mu\text{g L}^{-1}$  in freshwater environments. The lower concentrations tested in this study (0.1 and 1  $\mu\text{g mL}^{-1}$ ) fall within the range of heavily contaminated sites, while the higher concentrations (10 and 100  $\mu\text{g mL}^{-1}$ ) substantially exceed currently reported environmental levels. Several factors suggest that actual environmental NP concentrations may be underestimated: (1) current detection methods have limited sensitivity for sub-100 nm particles,<sup>38</sup> and (2) continued growth in plastic production and environmental accumulation may lead to elevated concentrations in the future.<sup>39</sup> Conversely, environmental NP concentrations may be overestimated due to analytical limitations in distinguishing plastic from other nanoparticles<sup>40</sup> and laboratory contamination during sample processing.<sup>41</sup> Given these methodological uncertainties in environmental NP quantification, the lack of significant effects at lower concentrations in this study should be considered in the context of environmental risk assessment.

Although MP studies have demonstrated that the particle shape affects toxicity outcomes, direct evidence for shape-dependent toxicity at the nanoscale remains limited. The present study used fragmented NPs but did not include a direct comparison with spherical particles under identical experimental conditions. Therefore, it is not possible to determine whether the observed effects are attributable to particle shape or other factors such as polymer type and particle size. In addition, the toxicity mechanisms were not investigated in the present study. Future studies should address these limitations to better understand NP toxicity in aquatic organisms.

## 5. Conclusions

This study provides chronic toxicity data for PE NP fragments using well-characterized particles with food levels at 0.2 mg C  $\text{L}^{-1}$ . In a 27-day exposure study with *D. magna*, we found that PE NP fragments caused no mortality but induced significant sublethal effects, including reduced offspring production (10

and 100  $\mu\text{g mL}^{-1}$ ), decreased adult body length (10 and 100  $\mu\text{g mL}^{-1}$ ), and diminished population growth rates (1, 10, and 100  $\mu\text{g mL}^{-1}$ ). These findings demonstrate that PE NPs can impair life-history parameters and population growth rates of *D. magna* inhabiting environments with limited food availability, where concentrations of PENPs are higher than 1  $\mu\text{g mL}^{-1}$ . These concentrations substantially exceed currently reported environmental levels, and the sublethal effects should be interpreted within this context. Future investigations should examine cellular and molecular mechanisms and consider environmentally relevant concentration ranges.

## Author contributions

Jinyoung Song: investigation, data curation, validation, writing – original draft. In Young Kim: investigation, data curation, validation. Seonae Hwangbo: formal analysis, methodology. Tae Geol Lee: conceptualization, supervision, writing – review & editing. June-Woo Park: supervision, writing – review & editing.

## Conflicts of interest

There are no conflicts to declare.

## Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

Supplementary information (SI) is available. See DOI: <https://doi.org/10.1039/d5va00329f>.

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