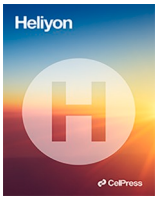
Heliyon 10 (2024) e40065

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Ecological fitness impairments induced by chronic exposure to polyvinyl chloride nanospheres in *Daphnia magna*

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ARTICLE INFO

Keywords:
Nanoplastics
Polystyrene
Polyvinyl chloride
Daphnia magna
Chronic effects
Fitness

ABSTRACT

The aim of this study was to evaluate the effects of chronic exposure (21 days) to an environmentally relevant concentration (10 μg/L) of two different nanoplastic (NP) polymers on the aquatic model organism *Daphnia magna*. This study examined the impact of exposure to 200 nm polystyrene nanoplastics (PS-NPs) and polyvinyl chloride nanoplastics (PVC-NPs), which had an average size similar to that of PS-NPs (ranging from 50 nm to 350 nm). The effects of polymer exposure on morphometric parameters, number of molts, swimming behaviour, and reproductive outcomes were evaluated. The findings indicate that PVC exposure induced higher body dimensions, while both polymers resulted in an increase in molting behaviour. Moreover, exposure to PVC-NPs had a negative impact on the reproduction of *D. magna*, as evidenced by a delay in the day of the first brood, a reduction in the total number of offspring produced, and, consequently, a slower population growth rate. It is hypothesised that the ingestion of PVC-NPs by *D. magna* may have resulted in an impairment of ecdysone hormone functionality and that the increased moulting events potentially representing an adaptive response to the negative effects of PVC-NP adhesion to the organism's body surfaces. These two organisms' responses could concur to explain the observed effects. This study identified the fitness impairments caused by exposure to PVC-NPs, which can lead to relevant ecological consequences. The comparative analysis of the effects induced by two types of polymers has revealed the generation of disparate hazards to *D. magna*. Furthermore, the chemical composition appears to be a pivotal factor in the onset of these effects. It can therefore be stated that PS is not a suitable standard for representing the toxicity of all plastics.

1. Introduction

Plastics are considered ubiquitous contaminants [1] because of their constant disposal into the environment, which is enhanced by their global production, reaching nearly 400 million tons in 2022 [2]. In freshwater ecosystems, municipal wastewater treatment plants contribute significantly to the presence of plastics in the environment, derived from industrial wastewater, domestic wastewater, and rainwater [3–5]. Microplastics (MPs) can be defined as plastics from 5 mm to 1 μm in diameter, whereas nanoplastics (NPs) are those ranging between 1 nm and 1000 nm [6,7]. The size of plastics strongly affects their interactions with biota and the potential hazards they pose to ecosystems [8], with small plastics generally having a greater impact than larger ones [9,10].

Scientific research has largely focused on MPs [11,12]. The available data on their environmental concentrations and effects on

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https://doi.org/10.1016/j.heliyon.2024.e40065
Received 10 June 2024; Received in revised form 31 October 2024; Accepted 31 October 2024
Available online 5 November 2024

organisms have contributed to progress in assessing the risks of MPs [13]. In contrast, the understanding of the environmental concentrations and effects of NPs on biota is still limited [14–17]. The quantification of NPs in environmental matrices is currently hampered by methodological limitations, which present a significant challenge in assessing the environmental pollution levels of these particles [18,19]. Ecotoxicological studies investigating the hazards of NPs to aquatic organisms are scarce. The evaluation of the toxic effects of NPs has typically focused on engineered plastic nanospheres as substitutes for environmentally relevant NPs [20]. In this context, polystyrene (PS) is the only plastic polymer that can be easily synthesised in the nanometric size range [21]. Consequently, evaluation of the effects of other NP polymers is still in its infancy because of the lack of feasible synthesis protocols [22,23]. However, since a wide variety of plastic polymers are occurring in the environment [24,25], it is crucial to identify the specific hazard associated with each polymer to ensure appropriate risk assessment [26]. Moreover, the polymer type can influence plastic toxicity [27–29]. Consequently, the use of PS toxicity data as a proxy for all other NPs has recently been questioned [30].

From this perspective, polyvinyl chloride (PVC) is worth consideration because it is the third-most produced plastic polymer worldwide [2]. Furthermore, its presence in environmental matrices as NP has been recently confirmed [25,31], and it is considered a potential hazard for the environment [32–34].

The present study aimed to contribute to the production of evidence of the effects of PVC-NPs and PS-NPs on *Daphnia magna*, by evaluating the chronic effects resulting from a 21-day exposure to engineered PVC and PS nanospheres at environmentally relevant concentrations. The microcrustacean *Daphnia magna* is a freshwater species recommended by the Organization for Economic Cooperation and Development (OECD) as a model organism for toxicity testing of chemicals [35] and it is also considered a suitable model for studying the toxicity of NPs [23,36]. Chronic exposure more closely reflects the conditions experienced by *D. magna* in the environment than short-term exposure [37] and allows the investigation of chronic effects that are considered essential for the correct evaluation of NP toxicity [38]. However, only a limited number of studies have investigated the chronic effects of NPs on organisms. Moreover, most of these studies have focused on PS-NPs, whereas the impacts of other polymers at the nanometric scale remain largely unexplored [20].

A multiple endpoint approach was used to assess the potentially harmful effects of *D. magna* exposure. For each individual, the life-history traits of survival, growth, and reproduction were monitored until the end of the experiment. According to the dynamic energy budget theory [39], certain contaminants can affect an organism's uptake of food and its use of energy and nutrients for maintenance, growth, juvenile development, and reproduction. In addition, the modulation of swimming behaviour, which is considered a sensitive biomarker [40], was investigated. This approach allows for a broad evaluation of the potential impact of NPs on *D. magna*. The evaluation and comparison of the chronic effects induced by PS-NPs and PVC-NPs can help elucidate the role of polymers in the onset of hazards to ecosystems, providing useful data for better characterisation of the ecological risk of NPs.

2. Materials and methods

2.1. Procurement and characterization of nanoplastics

The PS-NPs used in this study were monodisperse nanospheres (nominal diameter200 nm) supplied at a nominal concentration of 25 mg/mL (Polysciences Europe GmbH, Germany). PVC-NPs were procured from the European Commission's Joint Research Centre (JRC, Ispra, Italy) and consisted of polydisperse nanospheres synthesised in a range of sizes between 50 and 350 nm at a concentration of 2 mg/mL, as described in the protocol proposed by Cassano et al. [41,42]. As reported by the suppliers, PS-NPs had a density of 1.05 g/mL, whereas that of PVC was 1.40 g/mL.

Both tested NP solutions were carefully characterised before the exposure experiments. A full and detailed description of the NP characterisation and quality control has been published in our previous work [29]. The spherical shape of the particles was confirmed by scanning electron microscopy and transmission electron microscopy, and the chemical characterisation of the polymer was assessed using confocal Raman spectroscopy. The nominal concentrations and sizes of the NPs were verified through nanoparticle tracking analysis. Moreover, the hydrodynamic size and ζ-potential of NPs in the testing sample water were assessed before starting the exposure and after 48 h through dynamic light scattering (DLS). The size of the NP suspension was also analysed by transmission electron microscopy; PS-NPs exhibited an average size of approximately 200 nm, whereas PVC-NPs showed a wider size distribution ranging from 50 nm to 350 nm, with a mean size comparable to that of PS-NPs. The ingestion and presence of NPs on the body surfaces of exposed *D. magna* individuals were confirmed using a test with fluorescent PS-NPs. Finally, because the release of Cl^- from PVC solutions can induce toxicity in organisms [43], ion chromatography analyses were conducted on the PVC-NP leachate, which showed the absence of chlorine release by the PVC-NPs. Transmission electron microscopy, scanning electron microscopy, Raman spectroscopy, and DLS intensity measurements of PS-NPs (Fig. S1), and PVC-NPs (Fig. S2) are reported in the Supplementary Data.

2.2. Daphnia magna population maintenance

D. magna Straus (1820) individuals, obtained from ephippia purchased from Microbiotest (Daphtoxkit F, DM586), were cultured at the University of Milano-Bicocca (Italy) according to OECD Test Guideline No. 211 [35]. A detailed description of *D. magna* population maintenance is reported in our previous study [29].

2.3. Daphnia magna chronic exposure assay

The 21-day reproduction tests were conducted in accordance with the OECD Test Guideline No. 211 [35], using third-generation

D. magna neonates less than 24 h old. Three treatments were simultaneously performed: PVC-NPs, PS-NPs, and a control treatment (CTRL) consisting of pristine water. Each treatment involved 12 replicates with one individual placed in a glass vessel filled with 25 mL of the exposure solution. For the treatments involving PVC-NPs and PS-NPs, a concentration of 10 μg/L of NPs was added to each vessel. The individuals were maintained under the same conditions as the breeding individuals and provided with ample food. The static renewal method was applied every 48 h in this study. Molts and newborns were monitored daily and released.

The population intrinsic growth rate (r_m, day^{-1}), was derived from the Euler–Lotka equation [44]:

$$\sum e^{-rx} l_{xm_x=1} \quad \text{(Eq. 1)}$$

where x is the age class (days; 0–n), l_x is the probability of survival at age x, and m_x is fecundity at age x.

At the end of the bioassays, morphometric and behavioural data were collected for each adult. Behavioural alterations were evaluated as detailed in our previous study [29] by measuring the distance travelled (mm), velocity (mm/s), acceleration (mm/s^2), and activity time (%).

Morphometric parameters were assessed after video recording. Individuals were photographed, and images were analysed using ImageJ software to measure body length and width. Body length was measured from the top of the head to the base of the spine [35], and body width was measured at the widest point (Fig. S3).

To assess the vitality of the offspring, 15 individuals (less than 24 h old) were randomly selected from the first brood of the CTRL, PS-NP, and PVC-NP groups. Offspring behaviour was recorded in the same manner as that of adults, and their swimming distance travelled was measured as an indicator of vitality.

2.4. Statistical analysis

One-way analysis of variance (ANOVA) was used to analyse the effects of NP exposure on the investigated endpoints. Each endpoint (body length, body width, number of molts, number and time of the first brood, number of broods, number of neonates, and swimming behaviour parameters) was considered a dependent variable, and the different treatments (CTRL, PS-NPs, and PVC-NPs) were used as predictors. The Shapiro–Wilk test was used to verify data normality. An ANOVA or *t*-test was used to analyse the effects of NP exposure on the investigated endpoints of the normally distributed data. If the data were not normally distributed, they were log-transformed before statistical analyses were conducted. In cases where data were not normally distributed after log transformation, the Kruskal–Wallis test was used. Statistical significance was set at $p < 0.05$. Tukey's or Dunn's post-hoc tests were used after ANOVA or Kruskal–Wallis tests, respectively, to detect significant differences between the treatments.

Pearson's or Spearman's correlation analysis was conducted to determine the correlation between different variables. Principal component analysis (PCA) was performed to integrate and highlight the associations among the investigated variables, with data scaled prior to analysis. Before proceeding with the PCA, variables with a high correlation value ($r > 0.9$) were discarded. PCA was conducted on a data matrix comprising 36 samples (rows) and 6 variables (columns). All the statistical analyses were conducted using R software, implementing "ggplot2", "ggfortify", and "corrplot" packages in R version 4.3.2 [45].

3. Results and discussion

The tests met the requirements specified in the OECD Test Guideline No. 211 [35], as the mortality observed in the CTRL treatment was below 20 %. The results of the ecotoxicological endpoints are presented in Table 1. According to the statistical test results, exposure to the different NP polymers elicited varying responses in *D. magna*.

Table 1
Morphological, physiological, survival, reproductive, behavioural, and population dynamic endpoints measured in *D. magna* individuals over 21 days of exposure to CTRL, PS-NP, and PVC-NP treatments. Data are reported as mean ± standard deviation (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ vs. CTRL).

Endpoint	Unit of measure	CTRL	PS-NPs	PVC-NPs
Morphological	Length (mm)	2.9 ± 0.2	2.9 ± 0.2	3.2 ± 0.1**
	Width (mm)	1.9 ± 0.1	1.9 ± 0.2	2.1 ± 0.1*
Physiological	Number of molts	6.0 ± 1.1	6.9 ± 1.6 *	7.3 ± 1.1**
Survival	Number of dead	0	0	0
Reproductive	Number of broods	3.0 ± 0.8	2.2 ± 1.3	0.83 ± 0.8***
	Time of the 1st brood (day)	10.8 ± 1.4	14.7 ± 4.9	18.4 ± 5.1***
	Number of offspring per parent	27.5 ± 18.4	25.9 ± 16.2	10.9 ± 11.4**
Behavioural	Velocity (mm/s)	12.8 ± 3.6	12.8 ± 4.2	12.3 ± 3.1
	Acceleration (mm/s^2)	215 ± 45.9	206 ± 38.2	210 ± 37.4
	Distance (mm)	313 ± 103	299 ± 125	297 ± 89
	Activity (%)	79.6 ± 12.0	72.1 ± 11.7	79.0 ± 10.0
Population dynamic	Population growth rate (day^{-1})	0.41 ± 0.22	0.30 ± 0.18	0.10 ± 0.10***

3.1. Morphological and physiological endpoints

Regarding morphological traits, individuals exposed to PVC-NPs exhibited a higher average length and width than those exposed to CTRL and PS-NPs. Statistically significant differences in length were observed between individuals in the PVC-NP and CTRL groups ($p = 0.0029$) and between PVC-NP and PS-NPs groups ($p = 0.0260$). Statistically significant differences in body size were confirmed for width, with differences observed between PVC-NPs and CTRL ($p = 0.0203$) and PVC-NPs and PS-NPs ($p = 0.0209$). However, no alterations in the width/length ratio were detected, with a width/length ratio of 1.5 as reported by Chen et al. [46].

Regarding PS-NP results, the absence of effects on morphometric parameters is in line with the findings of Liu et al. [47] after a 21-day exposure at 1 μg/L of 75 nm PS-NPs. However, Besseling et al. [48] and Pikuda et al. [49] have shown the onset of morphometric alterations in *D. magna* in response to PS-NP exposure only at high tested concentrations (mg/L) and Heinlaan et al. [50] after multigenerational exposures at low concentrations (μg/L). To the best of our knowledge, there is no evidence in the literature on the dimensional changes in *D. magna* in response to exposure to PCV-NPs. Kim et al. [51] reported morphological changes in the cladoceran *Moina macrocopa* when exposed to concentrations >100 μg/L of a mixture of micro and nanosized PVC, resulting in a decrease in dimensions. In contrast, for PVC-MPs (>10 μm), alterations in morphometric parameters have generally been ascribed to the toxic chemicals present in the plastic bulk materials rather than to the polymer itself [27,33].

Considering the physiology of *D. magna*, both NP treatments increased the number of molts (Fig. 1).

The molting rate of CTRL individuals was lower (0.29 ± 0.05 molt/day) than those of PS (0.33 ± 0.06 molt/day) and PVC (0.35 ± 0.05molt/day). Statistical analysis showed that molting behaviour was significantly stimulated by both PVC-NP and PS-NPs treatments compared to CTRL ($p = 0.0022$ and $p = 0.0208$, respectively).

The CTRL treatment displayed a stepped curve in the molting cycle, with intermolt periods lasting three days, which is consistent with the normal behaviour reported by LeBlanc [52]. In contrast, the two NP treatments exhibited a continuum in the molt cycle, resulting in shorter intermolt periods (Fig. S4).

The present study showed that exposure to PS-NPs stimulated molting behaviour, which contrasts with previous findings. Rist et al. [53] found no alteration in molting behaviour after a 21-day exposure to 100 nm PS-NPs at 0.1, 0.5, and 1 mg/L, whereas Pikuda et al. [49] reported a decrease in the number of molts at 50 mg/L of 200 nm PS-NPs. The different particle sizes and concentrations tested may have contributed to the observed differences.

With regard to the effects of PVC exposure, in addition to the observed increase in body size, a stimulation of moulting activity was also observed. In arthropods, the functions of ecdysteroids, in particular ecdysone, are related to both growth and molting differentiation [54]. *D. magna* undergoes periodic exoskeletal exfoliation throughout its lifespan, accompanied by continuous molting and growth [55], with ecdysteroids mediating this process [56]. The observed effects of PVC-NPs on the body dimensions and molting behaviour of *D. magna* may therefore indicate a potential impairment of the hormonal functionality of ecdysone. Owing to the lack of studies conducted on PVC-NPs and *D. magna*, it was considered opportune to compare the present data with the current evidence for PVC-MPs. A recent study showed that small-sized PVC-MPs (2 ± 1 μm) can cause an increase in the molting frequency of *D. magna* [57]. The authors suggested that the ingestion of small PVC-MPs might affect the expression of genes responsible for the molt cycle, specifically those related to ecdysone biosynthesis. However, Kim et al. [51] reported the opposite effect, with micro-nano PVC fragments causing an extension of the intermolt period in *M. macrocopa*.

In addition to NP ingestion, another factor that may have concurred to the observed change in the molting cycle of *D. magna* exposed to PVC is the difference in the physical and chemical properties between PVC and PS [29]. This difference may have increased the adhesion and burden of PVC on the body surfaces of *D. magna*, which could have stimulated the observed molting behaviour. Yip et al. [58] reported that PVC-NPs exhibited stronger surface interactions than PS-NPs, which supports the hypothesis of the present study. Moreover, it should be noted that the external surface of *D. magna* individuals may have a higher load because of the higher density of PVC compared with PS. Interestingly, previous studies on nanomaterials have reported that the adhesion of TiO_2 and SiO_2

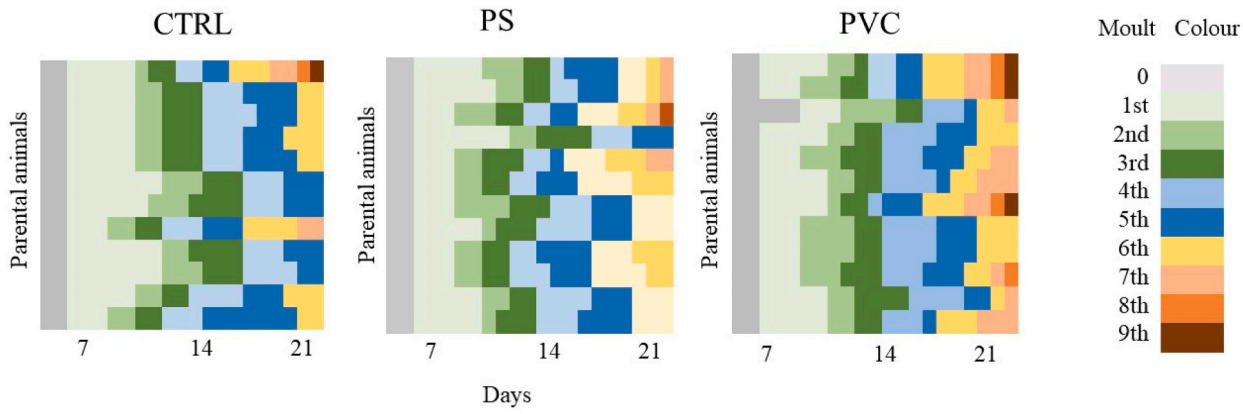

Fig. 1. Graphical representation of the number of molts produced by *D. magna* (12 individuals per treatment) over a 21-day exposure to CTRL, PS-NPs, and PVC-NPs. Each row corresponds to a surviving parent animal.

nanoparticles to the body surface of Daphnia can increase their specific weights. This can lead to alterations in the molting behaviour as a possible pathway for physical nanoparticle toxicity [59,60]. The authors proposed a causal relationship between the upregulation of genes related to molting (e.g., "*eip*") and the increase of ecdysone production, which accelerates the exfoliation of the exoskeleton to facilitates the shedding of the negative effects of nanoparticle adhesion. Similarly, the stimulation in molting behaviour for both NP treatments observed in the present study suggests that a possible pathway of NP toxicity could be related to biological surface coating. It is important to note that the main objective of this study was to evaluate the chronic effects of different NP polymers rather than to determine their specific mechanisms of action. Therefore, further analyses should be conducted to gain a better understanding of the role of physicochemical properties in the onset of the observed effects.

3.2. Reproductive endpoints

The effects on *D. magna* reproduction were assessed by monitoring the number of broods (Fig. 2), day of broods (Fig. S5), number of neonates produced in each brood (Fig. S6), and number of neonates produced over 21 days (Fig. 3).

The average number of broods generated under the CTRL treatment was 3.0 (±0.8), whereas for PS-NPs and PVC-NPs, the averages were 2.2 (±1.3) and 0.83 (±0.8), respectively (Fig. 2). Statistical analyses revealed a significant difference in the number of broods between PVC-NPs and CTRL ($p < 0.00001$), and between PVC-NPs and PS-NPs ($p = 0.0057$). In the CTRL group, the mean for the first brood was 10.8 days (±1.4), whereas in the PS-NP and PVC-NP groups, the means were 14.7 (±4.9) and 18.4 (±5.1) days, respectively. The delay of the day of the first brood observed for the PVC-NP treatment was statistically different from that of CTRL ($p = 0.0007$), whereas no statistically significant difference was observed between CTRL and PS-NPs. No statistical differences were found among the treatments with respect to the timing of the second, third, and fourth broods (Fig. S5).

In terms of the number of offspring produced per parent animal, CTRL individuals had an average number of 27.5 (±18.4) newborns per female, whereas parents exposed to PS-NPs and PVC-NPs produced 25.9 (±16.2) and 10.9 (±11.4) newborns, respectively. The average number of offspring in the PVC-NP group differed significantly from that in the CTRL ($p = 0.0089$) and PS-NP ($p = 0.0137$) groups. The cumulative curve of the number of offspring produced by the parent animals (Fig. 3) indicated a negative impact of NP treatments on *D. magna* reproduction. The number of offspring produced under each treatment was modelled by a second-degree polynomial regression, which provided a good fit for the CTRL ($R^2 = 0.9869$; $F = 413.2$; $p < 0.00001$), PS-NP ($R^2 = 0.9862$; $F = 393.4$; $p < 0.00001$), and PVC-NP ($R^2 = 0.9278$; $F = 70.65$; $p < 0.00001$) treatments.

In the CTRL treatment group, 50 % of all offspring ($n = 165$ newborns) were produced by day 15. However, the PS-NP group produced 88 newborns by day 15, requiring three more days to reach 165 newborns. In contrast, PVC-NP parents produced only 11 % ($n = 18$) of the offspring produced under CTRL by day 15. Moreover, the PVC-NP group, in contrast to the PS-NP group, did not reach the 50 % of the total offspring produced by the CTRL group; by the end of the test, they produced only 131 newborns in total. Notably, there was no statistically significant difference in the average number of neonates produced in each brood between the different treatments (Fig. S6). Therefore, the observed impairment in the fitness of *D. magna* under the PVC-NP treatment appeared to be primarily caused by a delay in the timing of the first brood, and a consequent decrease in the number of broods. PS-NP exposure resulted in an initial delay in newborn production without significantly affecting population growth. In contrast, the PVC-NP treatment impaired *D. magna* reproduction, leading to a significant reduction in the number of offspring produced.

At the current state of knowledge, the effects of NPs on reproduction in *D. magna* are still uncertain, with contrasting observations among different studies. Heinlaan et al. [50] reported the absence of an effect on the reproductive endpoints after multigenerational exposure to environmentally relevant concentrations of PS-NPs, whereas Monikh et al. [28] reported a decrease in the number of broods after a 21-day exposure. Although the absence of the effects induced by PVC NPs was recently reported [28], the results of other studies are consistent with those of the present study. Liu et al. [57] showed that small PVC-MPs (2 ± 1 μm) caused a delay in the day of the first brood and a decrease in the average number of offspring, whereas the exposure of the cladoceran *M. macrocopa* to PVC

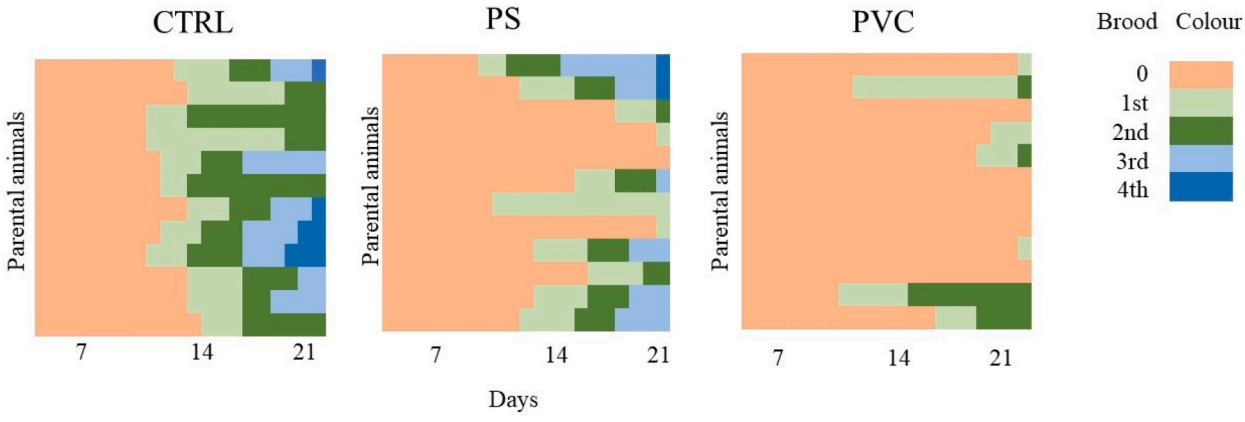

Fig. 2. Graphical representation of the number of broods produced by *D. magna* (12 individuals per treatment) over a 21-day exposure to CTRL, PS-NPs, and PVC-NPs. Each row corresponds to a surviving parent animal.

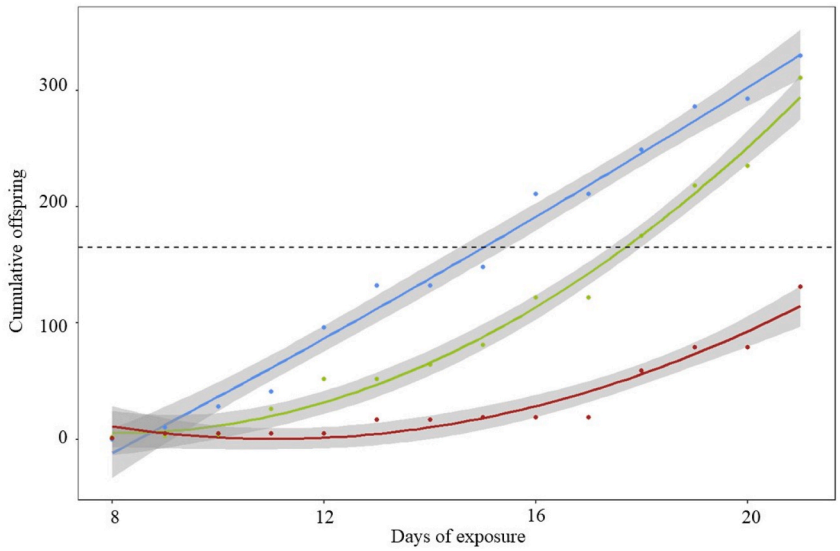

Fig. 3. Cumulative offspring curves of the twelve *D. magna* parents over 21 days for the CTRL (blue), PS (green), and PVC (red) treatments, obtained through a second-grade polynomial regression of experimental data (coloured dots). The black dashed line indicates the 50 % of the total offspring (n = 165) produced under the CTRL treatment. The shaded region represents the 95 % confidence interval for the true value.

fragments (ranging from 0.2 μm to 20 μm) led to inhibitory effects on reproductive fitness and population structure [51]. This suggests that PVC may have a long-term impact on the health of cladoceran populations. Notwithstanding, the mechanism of PVC-NP toxicity in reproduction remains largely unknown. It is retained plausible that the increased molting behavior and body dimension observed in PVC-NP exposure treatments may have concurred to a reduction of energy nutrients available for embryo development, leading to the observed effects on *D. magna* reproduction. The molting process plays a fundamental role in determining the growth and development of *D. magna* [61–63], and its cost is considered a major life-history constraint that can lead to an insufficient amount of energy to be spent for reproduction [64]. In this context, a recent article suggested that the increase in molting behavior and the decrease of offspring observed after exposure of *D. magna* to small PVC-MPs can be ascribed to alteration of energy metabolism [57].

3.3. Population dynamic endpoint

The population intrinsic growth rate (r_m), calculated using Eq. (1), is considered a suitable parameter for evaluating population health, because it is affected by the fecundity, lifespan, and developmental speed of organisms [65]. Fig. 4 shows the calculated r_m values for the three groups. Statistical analysis revealed significant differences in the r_m values between PVC and CTRL ($p = 0.0003$),

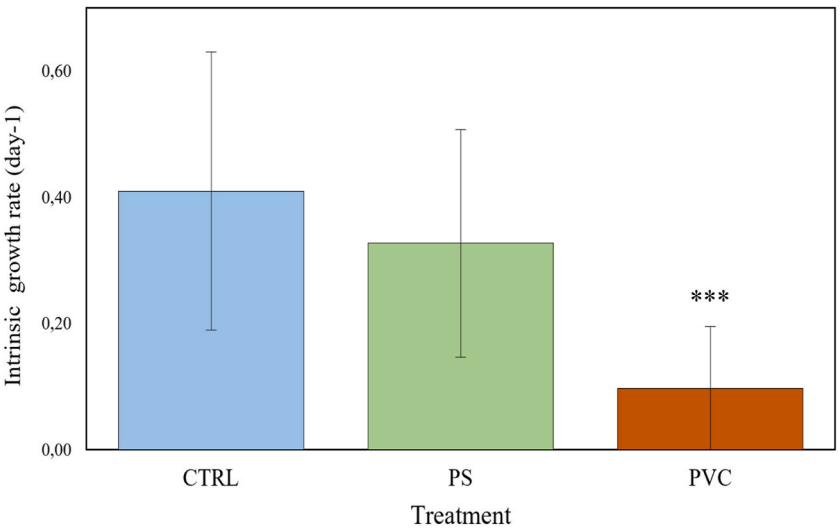

Fig. 4. Intrinsic population growth rate of *D. magna* after a 21-day exposure to CTRL, PS-NP, and PVC-NP treatments. (***$p < 0.005$ vs. CTRL).

and between PVC and PS ($p = 0.0162$).

The r_m in the CTRL group (0.41 ± 0.22 days^{-1}) is in line with previous studies [66,67]. Regarding PS-NP exposure, the results showed a lower r_m (0.30 ± 0.10 days^{-1}) than that for the CTRL group. A similar reduction in r_m was reported for *D. magna* in response to 100 μg/L of PS-MPs ranging from 1 to 5 μm [68]. In contrast, the PVC-NP treatment resulted in a strong decrease in reproductive performance, which was confirmed by a very low r_m (0.10 ± 0.10 days^{-1}).

These results suggest that exposure to PVC-NPs had a greater effect on the sexual maturity and reproduction of *D. magna* than exposure to PS-NPs. This was demonstrated by a significant delay in the day of the first brood, significant reduction in the average number of offspring produced, and sharp decrease in the intrinsic population growth rate.

3.4. Behavioural endpoints

Regarding behavioural traits, the evaluation of swimming behaviour did not reveal statistically significant differences among the different treatments (Table 1). Our previous study [29] reported that a shorter exposure time (48 h) to a comparable concentration (12.5 μg/L) of the two tested NP polymers did not induce alteration in the swimming behaviour of *D. magna* juveniles. Regarding PS-NPs, the absence of swimming alterations is in line with the evidence reported in the literature after 21 days of exposure [69,70]. Regarding PVC-NPs, our previous study showed that an exposure of 125 μg/L induced stimulation of swimming activity of *D. magna* juveniles [29]. The lack of effect observed in the present study could be attributed to the lower tested concentration (10 μg/L) and to the fact that *D. magna* juveniles are generally more sensitive to plastic exposure than adult individuals [71]. It is worth noting that the offspring vitality results shows that PVC individuals of the first brood moved less (69.5 ± 20.9 mm) than CTRL individuals (87.2 mm $\pm$ 23 mm) and those exposed to PS-NPs (77.9 ± 22.1 mm) (Fig. S7). Although statistical analyses did not reveal statistically significant differences, the observed trend suggests that the lower viability of first-brood PVC-exposed offspring. Individuals exposed to PVC-NPs exhibited increased size, significant stimulation of molting behavior, and a decline in the number of individuals produced. This suggests that the potential imbalance in the hormone functionality of ecdysone may have concurred in allocating more energy towards growth, with a subsequent reduction in reproductive investment. Consequently, the reduced viability of PVC-NP newborns could be ascribed to a decrease in energy resources allocated for reproduction by parental individuals.

3.5. Correlation among the different treatments and the investigated variables

Correlation analysis between the investigated parameters (Fig. 5a) showed that the number of broods was strongly negatively correlated with the time of the first brood ($r = -0.75$, $p < 0.001$), and positively correlated with the number of offspring ($r = 0.81$, $p < 0.001$). Surprisingly, the time of the first brood showed a positive correlation with the length of the individuals ($r = 0.65$, $p < 0.001$), indicating that the individuals with a delay in the time of the first brood have greater dimensions.

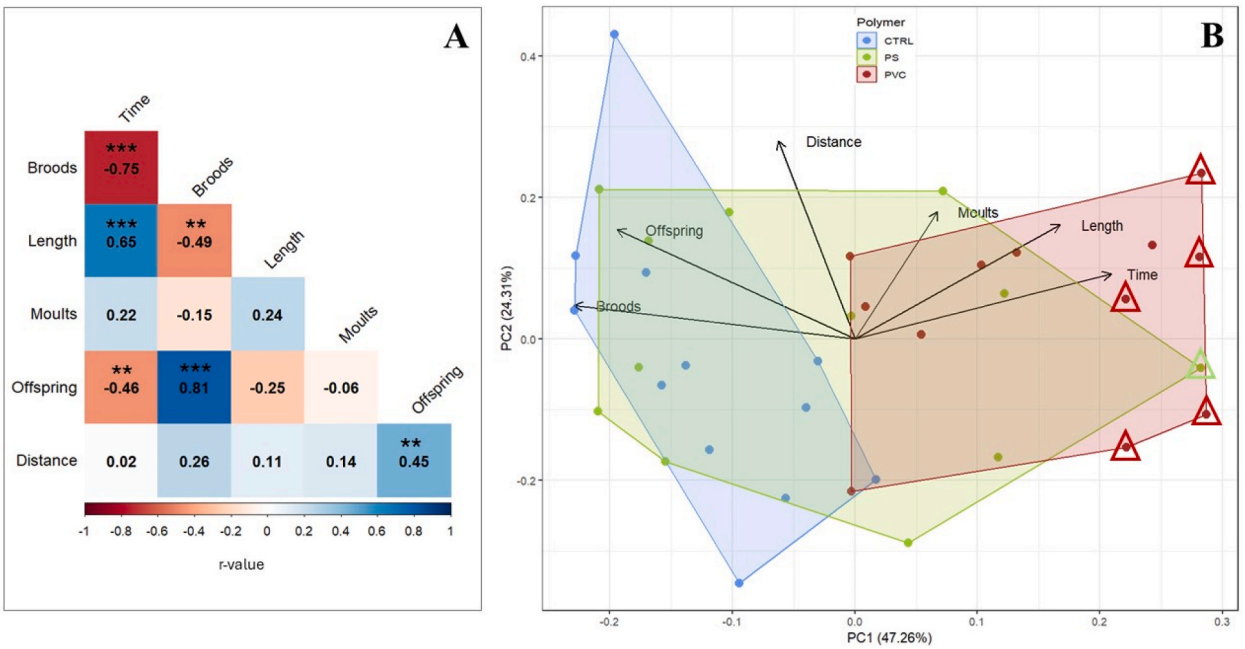

Fig. 5. Correlation plot (A) of *D. magna* number of broods, number of offspring, swimming distance, number of molts, length and time to the first brood (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$). Principal component analysis (B) of the effect of exposure conditions (CTRL, PS-NPs, and PVC-NPs) on *D. magna* based on the investigated parameters. Dots surrounded by triangles correspond to organisms that never reached sexual maturity.

PCA yielded two principal components (PC1 and PC2) that collectively explained 71.5 % of the total variation (Table S1). PC1 accounted for 47.6 % of the total variance and exhibited positive loadings with time of the first brood (r = 0.85) and length of *D. magna* (r = 0.68), as well as negative loadings with the total number of offspring (r = -0-89) and number of broods (r = −0.93). PC2 accounted for 24.3 % of the total variance, with all variables exhibiting positive loadings, particularly swimming distance (r = 0.73). The relationship between the two principal components and the analysed variables is illustrated in Fig. 5b, and detailed PCA correlation results are presented in Table S2.

As shown in Fig. 5b, organisms subjected to the CTRL and PVC-NP treatments were grouped into two distinct systems, whereas those from the PS-NP treatment group showed an overlapping distribution between these two groups. These results indicate that the negative effects of PVC-NPs are attributed to variables with high positive loadings with PC1, namely day of first brood, and body dimensions. Specifically, at the right end of the PC1 axis, a subgroup of the PCV-NP treatment was identified (dots surrounded by a triangle), corresponding to organisms that never reached sexual maturity. These were large organisms (Fig. 5b).

4. Conclusions

The impact of NPs on organisms is complex because of their presence in the environment as a mixture of plastic polymers of varying chemical compositions, shapes and sizes. The data presented here provide new evidence on the role of polymer type in toxicity. The different effects observed for PS and PVC treatments suggest that the chemical composition of the two investigated polymers may have contributed to their onset. It has been hypothesised that the ingestion of PVC-NPs by *D. magna* individuals had impaired the hormonal functionality of the ecdysone, leading to increased molting activity and larger body dimensions. It has also been suggested that the adhesion of PVC-NPs to the body surfaces of *D. magna* may have concurred to the stimulation of molting activity, thereby accelerating exoskeleton exfoliation to facilitate the elimination of the negative effects of PVC-NP adhesion. Consequently, it can be hypothesised that the increase in molting behaviour and body dimensions observed in the PVC exposure treatments may have led to a reduction in the energy nutrients available for reproduction, thus contributing to the observed ecological fitness impairment, in particular the delay in reaching sexual maturity.

In addition to the contribution of chemical composition, the role of size differences of the particles used in this study cannot be excluded, as PCV-NPs used had a slightly different size distribution than the PS-NPs.

The data presented here provide new evidence on the role of nanoparticle polymer type in toxicity. This study observed the effects of PVC on various endpoints at both the individual and population levels. PVC-NPs affected the *D. magna* population molting behaviour, morphometric parameters, and reproductive fitness over a prolonged period. In particular, the correlation between higher dimensions and lack of reproduction suggests the impairment of hormonal systems linked to ecdysone. From an ecological perspective, the observed delay in reaching sexual maturity could have a significant effect on *D. magna* populations in real ecosystems. Therefore, further analyses are recommended to assess the potential toxicity of this polymer. Based on the evidence reported here, it can be concluded that PVC-NPs has a greater environmental impact than PS-NPs. This information is useful for assessing the environmental effects of the emerging pollutants.

It is recommended that future studies consider the potential toxic effects of other NP polymers with higher global production, including low/high-density polyethylene (L/HDPE) and polypropylene (PP). In addition to the engineered nanospheres, it would be useful to study the non-engineered ones, such as fibres and irregular fragments. Furthermore, new types of biodegradable plastics, such as polylactic acid (PLA), represent a field that warrants further investigation. Moreover, it would be beneficial to investigate materials of greater complexity, such as products containing plastics, including tire-wear particles, cigarette butts, and clothing. It is also important to acknowledge the potential risks associated with the organic additives and inorganic co-formulants present in their mixtures.

Given that organisms in the environment are subjected to long-term exposure that far exceeds the 21 days generally foreseen by international guidelines, there is a clear need for multigenerational studies to provide data on NP effects that more closely reflect those occurring in natural environments. In this context, it is endorsed the proposal to update the current OECD No. 211 guideline to a duration of 28/30 days [72] to generate data that are more environmentally relevant. It is also encouraged the production of more ecologically relevant data regarding the toxicological direct and indirect effects induced by NPs at different levels of the bio/ecological hierarchical scale.

CRediT authorship contribution statement

Andrea Masseroni: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Lorenzo Federico:** Writing – original draft. **Sara Villa:** Writing – review & editing, Writing – original draft, Supervision, Resources, Investigation, Conceptualization.

Data availability statement

Data will be available on request, for more information please contact corresponding author.

Funding

This work was supported by the University of Milano-Bicocca [n. Rif. Int.: 2021-ATE-0292].

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.The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Sara Villa reports financial support was provided by University of Milano-Bicocca. Sara Villa reports a relationship with University of Milan-Bicocca that includes: employment, funding grants, and non-financial support. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors wish to extend their gratitude to Dr. Valsesia Andrea and Dr. Cassano Domenico from the European Commission Joint Research Centre (JRC, Ispra, Italy) for kindly providing the nanoplastics used in this research. Furthermore, we would like to acknowledge the valuable contribution of the students from the Master's course in Environmental Science at the University of Milano Bicocca during the experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e40065.

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