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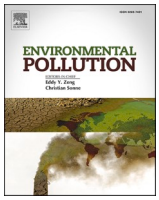

Sublethal effects induced by different plastic nano-sized particles in *Daphnia magna* at environmentally relevant concentrations☆

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ABSTRACT

A growing number of studies have reported the toxic effects of nanoplastics (NPs) on organisms. However, the focus of these studies has almost exclusively been on the use of polystyrene (PS) nanospheres. Herein, we aim to evaluate the sublethal effects on *Daphnia magna* juveniles of three different NP polymers: PS-NPs with an average size of 200 nm, polyethylene [PE] NPs and polyvinyl chloride [PVC] NPs with a size distribution between 50 and 350 nm and a comparable mean size. For each polymer, five environmentally relevant concentrations were tested (from 2.5 to 250 μg/L) for an exposure time of 48 h. NP effects were assessed at the biochemical level by investigating the amount of reactive oxygen species (ROS) and the activity of the antioxidant enzyme catalase (CAT) and at the behavioral level by evaluating the swimming behavior (distance moved). Our results highlight that exposure to PVC-NPs can have sublethal effects on *Daphnia magna* at the biochemical and behavioral levels. The potential role of particle size on the measured effects cannot be excluded as PVC and PE showed a wider size range distribution than PS, with particles displaying sizes from 50 to 350 nm. However, we infer that the chemical structure of PVC, which differs from that of PE of the same range size, concurs to explain the observed effects. Consequently, as PS seems not to be the most hazardous polymer, we suggest that the use of data on PS toxicity alone can lead to an underestimation of NP hazards.

1. Introduction

Nanoplastics (NPs) are particles sized between 1 and 1000 nm (Gigault et al., 2018; Hartmann et al., 2019) and are mainly formed by the environmental fragmentation of large plastic items (Gigault et al., 2021). Many ecotoxicological assessments utilize engineered nanometric plastics instead of naturally occurring ones in their bioassay. For enhanced terminological and experimental consistency, it is advised that the origin of plastics used in bioassay is explicitly stated. In comparison to primary particles present within commercial products, particles that enter ecosystems can deviate considerably in dimensions and physical-chemical characteristics (Lowry et al., 2012; Mitrano et al., 2015; Nowack and Mitrano, 2018). While several groups endeavor to create environmentally meaningful nanoparticles (Baudrimont et al., 2020; McColley et al., 2023), scrutinies of the ecotoxicological influence of such entities have usually investigated engineered plastic nanospheres (also referred to as primary NPs) as a substitute for the secondary plastic elements detected in the surroundings (Pikuda et al., 2023).

Recently, NPs have generated increasing scientific interest (Cerasa et al., 2021) owing to the unique properties that make them potentially more hazardous than microplastics (MPs) (Gigault et al., 2021). Different studies have demonstrated that NPs can negatively affect organisms (Shen et al., 2019) at the subindividual (Della Torre et al., 2014; Corsi et al., 2020; Liu H. *et al.*, 2020a,b) and individual levels (Mattsson *et al.*, 2015; Rist et al., 2017; Qu et al., 2019; Liu Z. et al., 2019). However, the environmental risk assessment (ERA) of NPs (NP-ERA) is in its infancy. The classical risk assessment framework requires the assessment of exposure and effect to determine whether the expected level of risk can be considered acceptable. The occurrence of NPs in

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ecosystems was confirmed in 2017 in ocean water (Ter Halle et al., 2017); however, recently, only a few studies have verified their presence in other abiotic matrices and biota, suggesting that NPs are present in the environment at low levels (Materić et al., 2021; Zhou *et al.*, 2021a,b; Materić et al., 2022a,b; Xu et al., 2022; Li et al., 2022). Comparing environmentally measured concentrations with data from ecotoxicological studies that tested similar concentrations, it was recently suggested that exposure to these environmental levels of NPs can be considered hazardous to aquatic ecosystems (Masseroni et al., 2022). However, the lack of standardized test materials for NPs makes assessing the effect of environmentally relevant concentrations of NPs challenging (Koelmans et al., 2022; Mitrano et al., 2023), thus leading to knowledge gaps that hamper NP risk characterization. To date, almost all studies have tested the effects induced by polystyrene NPs (PS-NPs) (Masseroni et al., 2022); this is because PS is the plastic polymer that is most easily processed into nanoparticles (Lehner et al., 2019). In the environment, NPs are present as a complex mixture of different polymers with varying sizes, shapes, surface functionalities, additive compositions, and degrees of weathering (Raynaud et al., 2021); therefore, the toxicity data of PS-NPs cannot be considered a reliable proxy for all other polymers (Monikh et al., 2022). There are very few effect assessment reports of other NP polymers whose presence on the market is very diffuse (e.g., polypropylene [PP], polyethylene [PE], and polyvinyl chloride [PVC]) (Kokalj et al., 2021; Plastics Europe, 2022). This study aims to address this gap by evaluating the sublethal effects of different NP polymers (PS, PE, and PVC) on the freshwater cladoceran *Daphnia magna,* setting the NP concentrations to environmentally relevant levels (lower than 250 μg/L), and the exposure time to 48 h. Even if this species is not the most sensitive one (Masseroni et al., 2022), it is the most frequently used model species in ecotoxicology (Qiao et al., 2022) and, as a filter-feeding zooplankton, it is representative of primary consumers of the freshwater food chain (Kukkola et al., 2021). As the selected concentrations are far below those inducing mortality, we have evaluated the effects of NPs at the subindividual and individual levels of biological organization, considering biochemical and behavioral endpoints.

In summary, this study aims to provide crucial data that will help establish whether different NP polymers exhibit comparable toxicity to PS-NPs and whether PS-NPs can be used as a proxy for all other NP polymers.

2. Materials and methods

2.1. Procurement of nanoplastics

Three polymers (PS, PE, and PVC) that best represent the most common plastic polymers present on the market and in the environment (Plastics Europe, 2022) were selected for this study. PS-NPs (200-nm nominal diameter, 25 mg/mL) were purchased from Polysciences Europe GmbH, Germany. PE-NPs and PVC-NPs were procured from the European Commission's Joint Research Center in Ispra, Italy, where they were synthesized following the protocol proposed by Cassano et al. (2021, 2023), with sizes ranging between 50 and 350 nm. As reported by the suppliers, the polymer density was 0.92 g/mL for PE (Sigma Aldrich, Milan, Italy), 1.4 g/mL for PVC (Sigma Aldrich, Milan, Italy), and 1.05 g/mL for PS (Polysciences Europe GmbH, Germany).

2.2. NP characterization and quality control

The three investigated solutions of PS-NPs, PE-NPs and PVC-NPs were well characterized before the exposure experiments.

NP shape: Transmission electron microscopy (TEM, JEM-2100, JEOL Ltd., Rome, Italy) and scanning electron microscopy (SEM, FEI Nova NanoLab 600 DualBeam, Thermo Fisher Scientific, Delft, The Netherlands) analyses were performed to check the morphology of the polymeric particles. In brief, stock suspensions were diluted in Milli-Q water and manually deposited on Formvar carbon-coated 200 mesh copper grids (Agar Scientific, London, UK) for TEM analysis at 120 kV, and on silicon wafer for SEM analysis.

Chemical characterization of NPs: To accomplish the chemical characterization of the NP polymers, Raman spectral analyses were performed using an alpha300 R confocal Raman microscope (WITec, GmbH, Berlin, Germany). In brief, stock suspensions were manually deposited (10 μL) on silicon wafer and analyzed by Raman microscope equipped with a 532 nm laser. Spectra were collected using the 50X objectives by averaging at least 10 spectra. The identification of the spectra was achieved by comparing the PS-NPs, PVC-NPs, and PE-NPs with the respective reference polymers.

NP concentration and size: Each polymer suspension was subjected to nanoparticle tracking analysis (NTA, NanoSight NS300, Malvern Panalytical, Worcestershire, UK) to verify the nominal size and concentrations of the NPs. In brief, the NP suspensions were diluted with Milli-Q water to achieve a concentration within 10^8 particles/mL, and then each polymer suspension was analyzed in triplicate (three 60-s videos were recorded). The nominal concentration (particles/mL) of the stock solution of PS-NPs was provided by the supplier, whereas for PVC-NPs and PE-NPs this was calculated using the following formula:

$$Concentration = \frac{6W \times 10^{12}}{\rho \times \pi \times \varphi^3}$$

where W is the grams of polymer per mL in latex, ρ is the density of the polymer, and φ is the diameter in microns of latex particles.

As reported in recent studies (Pikuda et al., 2019; Vaz et al., 2021; Kelpsiene et al., 2022), to avoid the unexpected effects of surfactants on organisms during ecotoxicology assays, it is essential to purify the synthesized NPs by means of a washing procedure. The PS-NP solution, given the presence of residual surfactants in the suspension, was washed using gentle sonication and centrifugation (13,500 rpm for 13 min), and then, the pellet was resuspended in Milli-Q water. NTA was also carried out for the washed PS-NP solution to identify any changes in the solution characteristics occurring as a consequence of the washing process. For PVC- and PE-NP suspensions, this step was not required because the synthesis protocols involved the use of biocompatible sodium cholate surfactant (Cassano et al., 2021; Cassano et al., 2023), presents in the highest NP tested exposure at a concentration of 0.0625 mg/L. Data from leachate experiment support the lack of detrimental effect induced by sodium cholate (refer to paragraph 2.2: *Additional quality control for PVC-NPs in the exposure water*).

Furthermore, the size distribution of the investigated particles was assessed through TEM imaging acquired at 120 KV. Images of 200 individual particles from each sample (PS-NPs, PVC-NPs, and PE-NPs) underwent manual analysis, with data being collected via ImageJ software. MinFeret (nm) size distribution, corresponding cumulative percent curve and Gauss fit (Xc; sigma) were obtained by OriginPro (2015) software. Mean and corresponding standard deviation of MinFeret (nm) were calculated by excel.

NP surface charge: To investigate the stability of the particles in suspension, their hydrodynamic size and ζ-potential were measured through dynamic light scattering (DLS) analyses using a Zetasizer Nano ZS instrument (Malvern Panalytical, Worcestershire, UK) equipped with a 633-nm helium–neon (HeNe) laser. Each polymer suspension was diluted to a concentration of 10^9 particles/mL and analyzed both in Milli-Q water and in the testing sample water. Three consecutive measurements (both for hydrodynamic size and ζ-potential) were carried out at T0 and T1 (48 h). Since it is plausible that the surface charge changes as soon as the plastic particles bind to molecules excreted by the daphnids, we have performed further DLS measurements to mimic the behavior of the investigated polymers in the exposure water. Briefly, we left the daphnids in the exposure water for 48 h, then removed the organisms from the NPs polymer suspension, which was analyzed by DLS as described above.

NP ingestion: A preliminary test was performed to confirm the

effective ingestion of NPs by *D. magna* juveniles. In brief, *D. magna* juveniles were exposed to 20-nm fluorescent PS-NPs (F8787 FluoSpheres, Invitrogen, Thermo Fisher Scientific, USA) at a concentration of 1000 μg/L for 48 h. At the end of the exposure, the presence of NPs in the digestive tract and on the body surface was investigated using point scanning confocal microscopy (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany).

Additional quality control for PVC-NPs in the exposure water: Since it is known that Cl^- and PVC monomers can induce toxicity in *D. magna* (Al-Malack et al., 2000; Lithner et al., 2012), we conducted further analyses on PVC-NPs leaching into the exposure water. In brief, PVC-NPs at the highest tested concentration (250 μg/L) were allowed to leach into the exposure water for 48 h. The release of Cl^- was investigated using ion chromatography (Thermo Scientific™ Dionex™, Waltham, MA, USA) and the Cl^- levels in the leachate were compared with those in pristine exposure water solution, as described by Nava et al. (2020). Finally, to exclude the role of leachate in influencing changes in the swimming behavior of *D. magna* individuals, the PVC-NP suspension was centrifuged (13,500 rpm for 15 min) and the supernatant (the water phase containing the leachate) was separated from the pellet (containing precipitated PVC-NPs); then individuals were exposed to the supernatant.

2.3. Daphnia magna population maintenance

The test organisms were derived from a single parthenogenetic female *D. magna* Straus, 1820, obtained from the Istituto Superiore di Sanità (Rome, Italy), and they were cultured by following Test Guideline 202 (OECD.). In brief, 20 *D. magna* individuals were bred in a glass beaker filled with 500 mL of a 1:1 mixture of two commercial mineral water samples (San Benedetto®—conductivity: 428 μS/cm at 20 °C; pH: 7.55; constitution: 283-mg/L HCO^{-3}, 51.1-mg/L Ca^{2+}, and 29.9-mg/L Mg^{2+}; San Bernardo®—conductivity: 52 μS/cm at 20 °C; pH: 7.00; constitution: 32.3-mg/L HCO^{-3}, 48.6 mg/L Ca^{2+}, and 0.39-mg/L Mg^{2+}), also referred to as exposure water throughout this article. The beakers were placed in a thermostatic chamber under a 16:8 light–dark cycle at a controlled temperature of 20.0 °C ± 0.5 °C. The daphnids were fed three times a week with a suspension of the commercial green algae spirulina (*Arthrospira platensis)* (1.25 mg/mL) and yeast (*Saccharomyces cerevisiae)* (10 mg/mL). The culture medium was renewed three times a week.

2.4. Daphnia magna exposure assay

48-h exposure assays were conducted with the third-generation neonates of *D. magna* (less than 24-h old). Once solution conditions were confirmed, the different NP suspensions were diluted with the exposure water to obtain solutions with a concentration of 250 μg/L; other subsequent concentrations (125, 25, 12.5, and 2.5 μg/L) were obtained via serial dilution. For each NP polymer, each concentration was tested to separate sets of 60 daphnid neonates. For each treatment, the samples were divided into 6 replicates, each containing 10 daphnids, and added into glass vessels containing 20 mL of the exposure solution. Control treatments (CTRL = treatment with the pristine exposure water) were performed in parallel. After the exposure, the organisms were collected for sublethal toxicity analysis.

2.5. Analyses of biochemical and behavioral biomarkers

For each NP treatment, the swimming activity of 30 daphnids was analyzed. Each individual daphnid was gently placed in a well plate containing 2 mL of exposure water. The plates were placed in a thermostatic chamber (20.0 °C ± 0.5 °C) equipped with a digital high-definition camera (Raspberry Pi 3 with Camera Module 2) set at a high resolution (1920 × 1080 pixels). After 5 min of acclimation, the swimming activity of the *D. magna* neonates was tracked through video tracking analysis using the software LoliTrack V4 (Loligo Systems, Denmark). The movement of each daphnid was recorded three consecutive times for 30 s (each video consisting of 750 frames at 25 frames/s). The distance traveled, expressed in mm, was chosen as the representative movement endpoint (Bownik, 2017). For the PVC-NP leachate experiment, a similar experimental set-up was used, with the different treatments (CTRL, leached PVC-NPs, and 250-μg/L PVC-NPs) being performed in parallel, each involving a total of 12 daphnids separated into 3 replicates of 4 individuals each.

For all the investigated polymers, the 30 daphnids analyzed for behavioral analysis and the other 30 were frozen at −80 °C for subsequent biochemical analysis. For each treatment, three replicates (pools of 18–20 organisms) were utilized. The amount of reactive oxygen species (ROS) and the activity of catalase (CAT) were investigated using spectrofluorimetry and spectrophotometry, respectively, in accordance with the procedures reported by De Felice et al. (De Felice et al., 2022).

2.6. Statistical analyses

The effects of NP exposure on the investigated endpoints were analyzed using one-way Analysis of Variance (ANOVA). Each endpoint was considered a dependent variable, while the treatments were considered the predictor. The normality of data was verified using the Shapiro–Wilk test, with the significance being set at a p-value <0.05. When data were not normally distributed, they were log transformed before being considered for ANOVA. Data not normally distributed even after log transformation were analyzed using the Kruskal–Wallis test. When ANOVA results were statistically significant, Dunnett's posthoc test was used to prove significant differences between the control and treatment groups. Conversely, Dunn's posthoc test was used for data analyzed using the Kruskal–Wallis test. Statistical analyses were performed using the R software (R Core Team, 2022).

3. Results and discussion

3.1. NP characterization and quality control

SEM, TEM, and Raman analyses confirmed the spherical shape and the chemical structure of the investigated polymers (Fig. 1).

The NTA results revealed that the particle size and concentration were within 15% and 30%, respectively, of the nominal values (Table 1). PVC and PE show a higher standard deviation in size measurements as they were synthesized in a 50–350 nm size range (Cassano et al., 2023). NTA results for particle number concentration are highly dependent on particle scattering characteristics, image acquisition, and analysis settings (Filipe et al., 2010; DeRose et al., 2022). In our study, the measured values were considered acceptable as they were within 30% of the nominal values. NTA verified that the PS washing step neither altered the relative size and concentrations of PS-NPs nor caused aggregation or loss of material (Fig. S1).

DLS size intensity measurements of PS-NPs, PVC-NPS, and PE-NPs in both Milli-Q and exposure water are reported in Table 2 and in Fig. S2. DLS size measurements in Milli-Q water were consistent with NTA size measurements. Polydispersity Index (PDI) results confirmed that PVC and PE particles exhibit a higher size distribution range than PS (Table 2). The ζ-potential values confirm the high stability of all the investigated polymers (Table 2). The ζ-potential is in fact considered a suitable indicator of NP surface charge (Bhattacharjee et al., 2014): ζ-potential values higher than |30| mV indicate a stable behavior of particles within the solution, whereas values between |10| and |30| mV suggest incipient stability (Martin et al., 2022). Moreover, surface charge is considered an important factor that can influence NP toxicity (Schwegmann et al., 2010; Sukhanova et al., 2018; Yu et al., 2019). The surface charge and size of NPs were also measured in the exposure water (both in presence and absence of *D. magna*) since these parameters can be influenced by the pH, salt content.

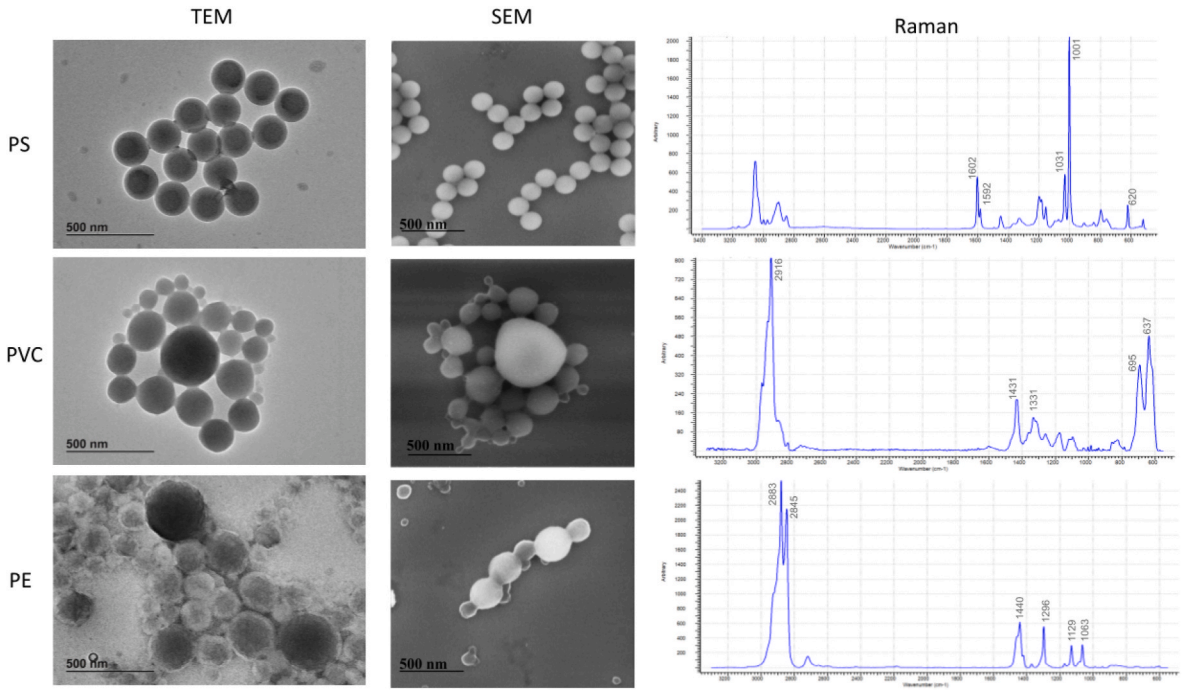

Fig. 1. Transmission electron microscopy (TEM), scanning electron microscopy (SEM) and Raman spectra of PS-NPs, PVC-NPs, and PE-NPs. The numbers in the Raman spectra specify the precise peaks utilized to identify PS, PVC, and PE polymers.

Table 1
NTA results for the size and concentration of the tested NPs. Data are presented as mean values of three consecutive measurements ± standard deviation.

Solution conditions	PS	Washed PS	PVC	PE
Nominal size (nm)	200	200	200	200
Measured size (nm)	182 (±32)	175 (±30)	229 (±65)	174 (±74)
Nominal concentration (particles/mL)	5.68×10^{12}	5.68×10^{12}	3.46×10^{11}	5.31×10^{11}
Measured concentration (particles/mL)	4.92 (±0.02) × 10^{12}	4.23 (±0.10) × 10^{12}	3.47 (±0.12) × 10^{11}	3.86 (±0.19) × 10^{11}

Table 2
DLS measurements of the hydrodynamic size (nm), PDI, and ζ-potential (mV) of the NPs in Milli-Q water and exposure water at T0 and T1 (48 h). Data are presented as mean values of three consecutive measurements ±standard deviation.

		Exposure time	Size	PDI	ζ-potential
PS-NPs	Milli-Q water	T0	214 ± 49	0.028	−33 ± 6
		T1	218 ± 48	0.011	−33 ± 6
	Exposure water	T0	199 ± 44	0.028	−32 ± 6
		T1	200 ± 51	0.033	−32 ± 7
PE-NPs	Milli-Q water	T0	227 ± 84	0.112	−38 ± 5
		T1	231 ± 86	0.114	−34 ± 5
	Exposure water	T0	207 ± 75	0.124	−31 ± 6
		T1	212 ± 86	0.125	−29 ± 6
PVC-NPs	Milli-Q water	T0	245 ± 85	0.164	−37 ± 5
		T1	246 ± 93	0.115	−36 ± 5
	Exposure water	T0	224 ± 108	0.167	−32 ± 6
		T1	277 ± 163	0.244	−25 ± 4

of the medium the NPs are suspended in (Auguste et al., 2020), and molecules excreted by *D. magna*. The ζ-potential and size values obtained for PS-NPs and PE-NPs showed that these parameters remained unaltered in the exposure water. In the case of PVC-NPs, slight changes in size and ζ-potential were detected in the exposure water as a function of time, with a decrease in the ζ-potential (from −31 to −25 mV) and an increase in the size (from 225 to 277 nm). The DLS measurements for NPs in the exposure medium with the presence of the molecules excreted by the daphnids (Table S1) suggest that the different polymers exhibit smaller surface charge values and slightly larger sizes, but with modest variations in size and charge between the three investigated polymers.

The size distribution obtained by TEM measurement shows that PS present an average size of 184.5 (±6.2) nm, while PVC and PE exhibit a distribution within the range of 50–350 nm, with an average size respectively of 116.7 (±65.0) nm and 145.5 (±39.5) nm (Fig. S3).

The results of the preliminary test with fluorescent PS-NPs (Fig. S4) confirmed in our test conditions the presence of NPs in the digestive tract and on the body surface of *D. magna* juveniles. These data are in line with the evidence provided in the literature, showing that *D. magna* can ingest different types of plastic polymers (Zimmermann et al., 2020) of both micrometric and nanometric size (Rist et al., 2017; Canniff and Hoang, 2018; Vighi et al., 2021; Jeyavani et al., 2023).

The ion chromatography results highlighted the absence of any difference in the chlorine content between the control exposure water and the PVC-NP leachates.

3.2. Effect assessment: biochemical biomarkers

Test results indicated that exposure to different NP polymers invoked different biochemical responses in *D. magna.* The tests were considered valid as they fulfilled the criteria elucidated in OECD Test Guideline 202 because during the exposure time the recorded mortality of daphnids under the control and test conditions was below 10% (Table S2). The detailed results of the statistical analyses are reported in the supplementary data (Table S3), and ROS and CAT histograms are presented in Fig. 2.

3.2.1. PS-NP exposure

In the PS-NP treatment group, no statistically significant differences were observed both for ROS levels ($p = 0.33$) and CAT activity ($p =$

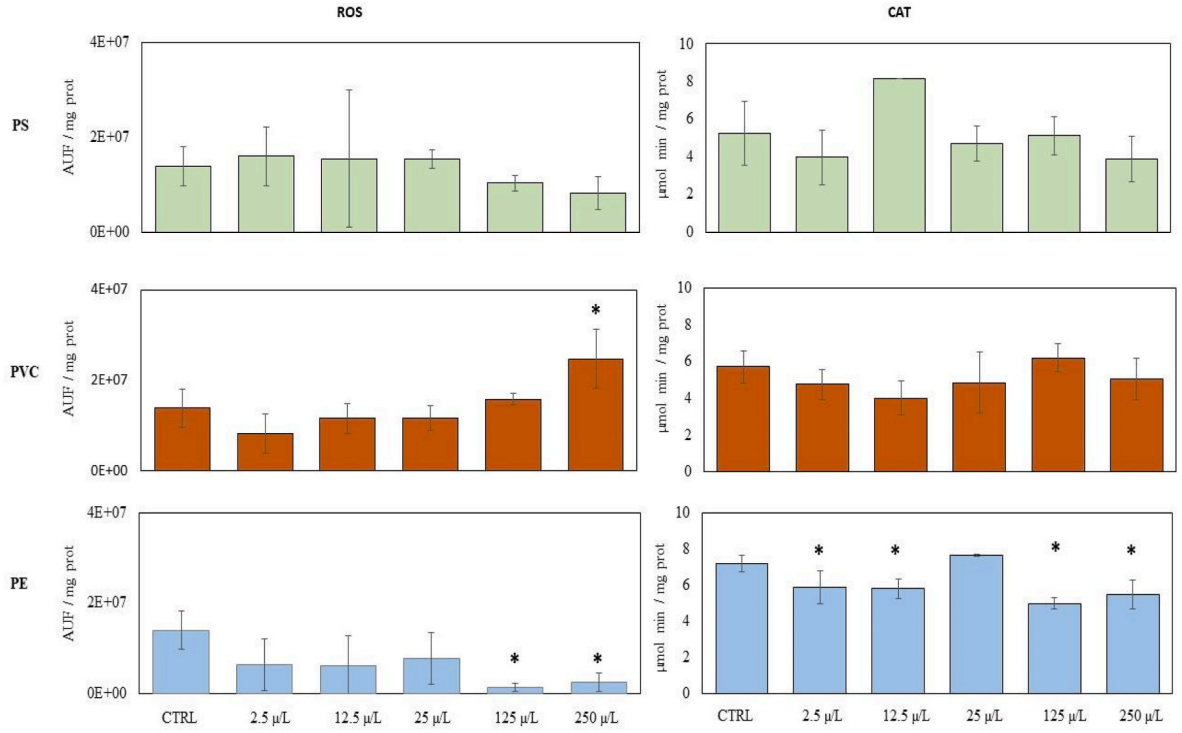

Fig. 2. ROS and CAT histograms measured after a 48-h exposure of *D. magna* to PS, PVC, and PE. * indicates statistically significant differences with respect to controls (p-value <0.05).

0.0934). The absence of PS-NP effects is consistent with results reported in the literature; in particular, in a recent study, *D. magna* individuals were exposed to PS-NPs (75 nm) for 21 d at concentrations comparable to those used in this study (50 and 500 µg/L), but they did not show signals of oxidative stress (De Felice et al., 2022). That said, as the response of an organism to NP exposure is strongly influenced by several factors that vary from one study to another (e.g., particle size, concentration, exposure time, and the model species involved), a straightforward comparison of our results with those reported in other studies is not possible. For example, the exposure of *D. pulex* to PS-NPs of 75 nm (100 µg/L) for 96 h resulted in the activation of antioxidant defense responses (Liu et al., 2018). This trend was further confirmed for an exposure time of 21 d using similar experimental conditions (Liu *et al.*, 2020a,b), even at lower concentrations of PS-NPs (Liu *et al.*, 2020c,b).

3.2.2. PVC-NP exposure

In the case of the PVC-NP treatment groups, significant differences were recorded in ROS levels at the highest tested concentration (250 µg/L, $p = 0.003$) in comparison to the controls, whereas no significant variations were detected in the CAT activity ($p = 0.253$).

A recent study reported alterations in the antioxidant system of *D. magna* after PVC-MP exposure (Liu et al., 2022). However, to the best of our knowledge, only one study has investigated the effects of PVC-NP exposure on *D. magna*, and this study established that PVC-NPs did not inflict any toxicity on the reproductive endpoints of the organism (Monikh et al., 2022). Evidence of ROS overproduction induced by PVC-NP exposure has been reported only for human cell lines (Mahadevan and Valiyaveettil, 2021).

The PVC-NP surface charge has been considered an important factor influencing PVC toxicity (Weber et al., 2022). In our study, we have observed that in the exposure scenario the measured surface charge of PVC presents modest differences when compared to those of PS and PE. This might indicate that the surface charge might not be the major vector of the observed effects.

Conversely, PVC-NP exposure was found to have no effects on the CAT activity in the daphnids. This dichotomy in the results of the two investigated biochemical endpoints could be attributed to the fact that 48 h of exposure, despite being enough to cause a surge in ROS levels, was not enough to induce the activation of the CAT activity. This result does not exclude that other proteins involved in the antioxidant machinery (for instance superoxide dismutase and glutathione peroxidase) might be induced in response to PVC-NP. Further analyses are therefore recommended, to assess the potential of this NP to impact on the antioxidant system and eventually induce oxidative damage.

3.2.3. PE-NP exposure

Our results revealed a sharp decline in the ROS content and CAT activity in daphnids for all the tested concentrations of PE-NPs (Fig. 2). With respect to ROS values, the two highest tested concentrations were statistically different from the control (125 µg/L, $p = 0.0008$; 250 µg/L, $p = 0.004$), a trend observed in the CAT histogram as well. The CAT activity results were statistically significant for all concentrations, except 25 µg/L (2.5 µg/L, $p = 0.046$; 12.5 µg/L, $p = 0.034$; 25 µg/L, $p = 0.754$; 125 µg/L, $p = 0.001$; 250 µg/L, $p = 0.008$). To date, the few studies that have evaluated the effects of PE-NPs on organisms (Baudrimont et al., 2020; Ekvall et al., 2022) have not considered oxidative stress endpoints. Alternatively, there have been studies that confirmed a PE-MP-mediated CAT activity decrease in oysters (Teng et al., 2021) and fish (Espinosa et al., 2019), suggesting that the polymer might hamper the smooth functioning of the antioxidant machinery. However, the authors of these studies suggest that an overproduction in ROS can overwhelm the antioxidant system, disrupting the activity of antioxidant enzymes such as CAT. Therefore, we would have expected an increase in ROS content and not a decrease. Further investigation of other oxidative stress biomarkers can provide useful information leading to a better understanding of the mechanism through which PE-NPs can induce an imbalance of the antioxidant machinery in *D. magna*.

3.3. Effect assessment: behavioral biomarkers

As in the case of biochemical biomarkers, treatment with different NPs of varying concentration invoked different swimming behavior from the *D. magna* juveniles (Fig. 3). The baseline swimming distance for the control groups was 77.7 (±26.5) mm, which is consistent with values reported in the literature (Pikuda et al., 2019; Bownik et al., 2020). The results of statistical analyses are reported in the supplementary data (Table S3), and the results pertaining to the swimming distance are reported in detail in Table S4. As seen from the results of the PVC-NP leachate experiment (Fig. S5), the leachate did not influence the swimming performance.

3.3.1. PS-NP exposure

It was observed that exposure to PS-NPs did not lead to changes in the swimming behavior of juvenile daphnids ($p = 0.496$). A few previous studies provide evidence of behavioral changes seen after a 48-h exposure to PS-NPs, but these studies used PS-NP concentrations several orders of magnitude higher than those tested in this study (Pikuda et al., 2019; Vaz et al., 2021; Nogueira et al., 2022). Behavioral changes (hopping frequency) were reported in a previous study (Pikuda et al., 2022) for longer exposure times and a higher concentration of 50,000 μg/L. Considering both the shorter exposure time and the lower tested concentrations used in this study, the absence of effects observed strengthens the evidence that PS-NPs do not induce behavioral alteration in *D. magna* at environmentally relevant concentrations, for both long (De Felice et al., 2022) and short exposure times.

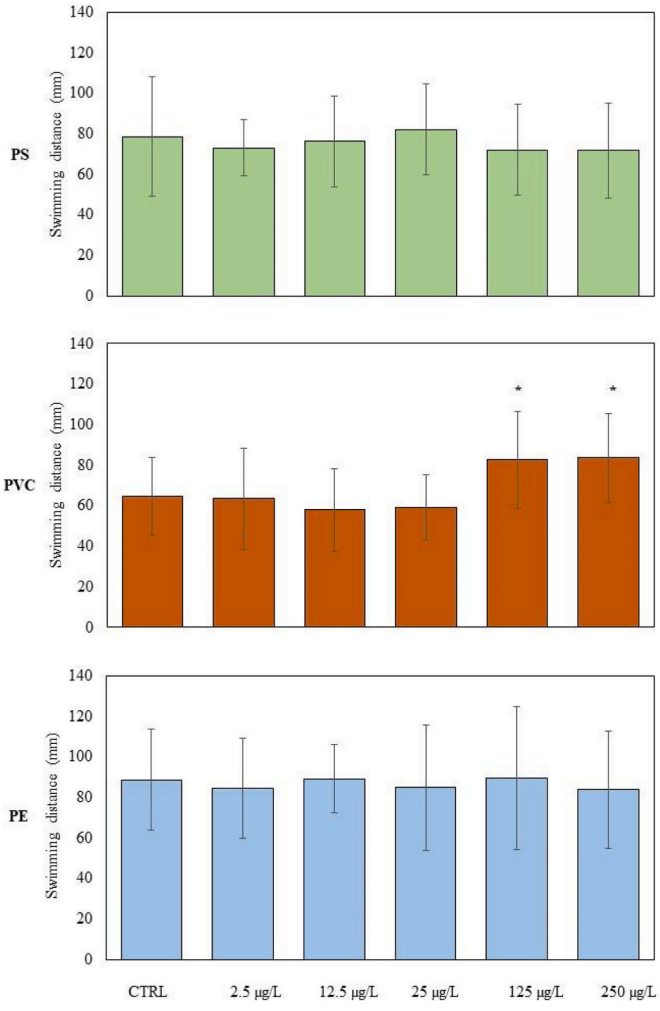

Fig. 3. Swimming behavior histograms showing the distance traveled by *D. magna* after a 48-h exposure to PS (a), PVC (b), and PE (c). * indicates statistically significant differences with respect to controls (p-value <0.05).

3.3.2. PVC-NP exposure

The distances traveled by *D. magna* juveniles after treatment with 2.5-, 12.5-, and 25-μg/L PVC-NPs were very similar to the control values; however, at the two highest tested concentrations, the juveniles exhibited increased swimming activity (125 μg/L, $p = 0.0335$; 250 μg/L, $p = 0.0138$). The PVC-NP leachate experiment further reinforced this stimulation in swimming activity caused by PVC-NPs ($p = 0.0198$), but, alternatively, it recorded no differences in the swimming activity between the leachate-treated juveniles and control group juveniles ($p = 0.9417$). Therefore, we conclude that the observed behavioral alterations are induced by PVC-NPs and not by the leachate released during the exposure.

The altered swimming behavior of daphnids draws attention to the potential hazards of this polymer to aquatic organisms. Behavioral alterations are considered ecologically relevant because they can potentially impact the ecosystem at a higher level (e.g., at the population and community levels) through a cascade of indirect effects (Saaristo et al., 2018). In particular, stimulation of swimming activity can alter competition ability and prey–predator interactions, leading to increased encounter rates with predators or making individuals less adept at escaping from them (Saaristo et al., 2018).

To the best of our knowledge, this is the first time that PVC-NP exposure–induced behavioral alterations in *D. magna* have been reported. This study has shown the adhesion of fluorescent-PS-NPs (20 nm) to the surface of the daphnid body and their presence in the digestive tract of *D. magna* juveniles (Fig. S4). Owing to the lack of fluorescent-PVC-NPs, the presence of PVC-NPs in the daphnids could not be verified, but daphnids exposed to PVC-NPs were found to retain reasonably similar behavior. In the literature, it is reported that the adhesion of nanoparticles (TiO_2 and SiO_2) to the external surface of *D. magna* can be considered a possible mode of inflicting physical nanoparticle toxicity (Dabrunz et al., 2011). The coating of the body surface of organisms with nanoparticles can result in increased specific weight and, in turn, swimming burden, leading to changes in the swimming behavior aimed at mitigating the negative effects of nanoparticle adhesion (Wang et al., 2021). In light of this, the changes in the swimming behavior observed after PVC-NP exposure could be ascribed to the physical properties and chemical structure of this polymer, which probably enhances its adhesion to the *D. magna* body surface as recently described by Yip et al. (2022). They report that PVC-NPs exhibit stronger surface interactions with the carapace of the acorn barnacle than PS-NPs. We are aware that this is a speculation and further investigations are required to better understand the observed effect, such as molecular changes in neurotoxicity biomarkers and SEM images of daphnids at the end of the exposure time.

Moreover, Weber et al. (2022) suggested that the density of PVC may be a contributory factor in its toxicity. The observations made in this study further corroborate this inference since PVC density (1.45 g/mL) is higher than those of PS (1.05 g/mL) and PE (0.92 g/mL), it may potentially have led to a higher burden on the body surface of *D. magna* juveniles, thus impacting their swimming behavior.

We underline that PVC-NPs (and PE-NPs) exhibit a higher size distribution than PS-NPs. The differences in size distribution, in particular the presence of particles smaller than 100 nm, could have played a role in the onset of the observed effects. In literature there is consensus on the importance of plastic size in causing hazard effects, with small plastic particles often tend to induce greater effect than large (Stock et al., 2020; Pochelon et al., 2021; Sendra et al., 2021). Unfortunately, due to the analytical issues in isolating PVC and PE particles larger than 100 nm, we have not tested PVC-NPs in the exact size range of PS-NPs. Therefore, we cannot exclude that the observed differences could derive from differences in the size distribution. However, comparing PVC data with those of PE (refer to paragraph 3.3.3.), we infer that other

factors, such as the surface chemistry of the different plastic polymers, might contribute rather than solely the size.

3.3.3. PE-NP exposure

PE-NP exposure did not induce behavioral alterations in the daphnids ($p = 0.808$). This result suggests that the modulation of ROS levels and CAT activity observed in *D. magna* exposed to this NP is not indicative of a condition of adverse sublethal effects that affects higher biological levels. It is plausible that, in contrast to PVC, an exposure time of 48 h was not enough for PE to induce behavioral alterations. Therefore, exposing *D. magna* to PE-NPs for longer periods of time (e.g., 21 d) could provide more effective information on the toxicity of this polymer on the organism.

By comparing the observed effects of different NP polymers with a similar particle size distribution, PE-NPs appear to be less toxic than PVC-NPs since at the individual level they don't induce changes in swimming behavior.

4. Conclusion

That different plastic particles induce different toxicity has been effectively demonstrated for MPs (Renzi et al., 2019; Zimmermann et al., 2020), whereas research is still in its infancy for NPs (Monikh et al., 2022; Weber et al., 2022; Li et al., 2023). This study established that environmentally relevant NP concentrations could inflict sublethal toxicity on *D. magna* and that these effects could differ with the various polymers tested. It further confirmed that sublethal endpoints, both at the biochemical level (oxidative stress endpoints) and the individual behavioral level (swimming distance), can be sensitive to even low concentrations of the NP contaminant. This study also suggests that the onset of sublethal effects after PVC exposure can be ascribed to the chemical structure and physical properties of this polymer, which are different from those of PE. However, the direct comparison between the toxicity of PS-NPs and the other two tested NP polymers is not straightforward due to the role of particle size on toxicity. Notwithstanding this, as PVC-NPs seem to induce a greater negative effect on *D. magna* than PE-NPs and given that they have particles of the same size, we have suggested that other factors might contribute to the toxicity rather than size only. The determination of the specific physical and chemical properties involved in the enhanced toxicity of PVC requires additional study. As the results in this study reveal that PS-NPs could not be the most hazardous NPs, it is crucial that the toxicity of other nanopolymers with different physicochemical properties be explored as well.

Credit author statement

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Declaration of competing interest

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

Data availability

Data will be made available on request.

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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.2023.123107.

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