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Research Paper

Chemical composition and particle size influence the toxicity of nanoscale plastic debris and their co-occurring benzo(α)pyrene in the model aquatic organisms *Daphnia magna* and *Danio rerio*

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ABSTRACT

Little is known about how particle chemical composition and size might influence the toxicity of nanoscale plastic debris (NPD) and their co-occurring chemicals. Herein, we investigate the toxicity of 3×10^{10} particles/L polyethylene (PE, 50 nm), polypropylene (PP, 50 nm), polystyrene (PS, 200 and 600 nm), and polyvinyl chloride (PVC, 200 nm) NPD and their co-occurring benzo(*a*)pyrene (BaP) to *Daphnia magna* and *Danio rerio*. During the 21 days of exposure to PE 50 nm and PS 200 nm, the number of broods produced by *D. magna* decreased compared to other treatments. Exposure to BaP alone did not produce any effects on the reproduction of the daphnids, however, the mixture of BaP with PS (200 or 600 nm) or with PE (50 nm) reduced the number of broods. Exposure of *D. rerio* embryos to PE 50 nm, PS 200 nm, and PS 600 nm led to a delay in the hatching. The presence of PS 200 nm and PVC 200 nm eliminated the effects of BaP on the hatching rate of zebrafish. Our findings suggest that data generated for the toxicity of one type of NPD, e.g. PVC or PS may not be extrapolated to other types of NPD.

1. Introduction

The commonly found types of plastics in the environment are polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), and polystyrene (PS) (de Sá et al., 2018). Plastics in the environment are fragmented into small pieces, because of various weathering processes (Huang et al., 2021). These processes can lead to the formation of microplastics (1 μm < size <5 mm) and nanoscale plastic debris (NPD, size <1000 nm). A considerable number of studies reported the adverse effects of microplastics and NPD in different ecosystems. For example, Brun et al. (2019) showed that PS-NPD induced disruption in glucose homeostasis coincided with increased cortisol secretion and hyperactivity in zebrafish larvae. It was also reported that PS-NPD enhanced the oxidative stress in *Daphnia pulex* (Liu et al., 2021) and decreased the activities of antioxidant enzymes in *Macrobrachium nipponense* (Li et al., 2020). Although some studies have already reported that microplastics other than PS (e.g. PP, PVC and PE) might also induce toxicity to aquatic organisms (Lithner et al., 2012; Zhu et al., 2019; Wang et al., 2020; An et al., 2021), such information is generally missing for NPD. Most of the available studies on toxicity of NPD have used only PS as a model for the particles. There are knowledge gaps on the acute and chronic toxicity of e.g., PVC, PE, and PP-NPDs.

The hazard of NPD is not limited to the particles themselves. Like their microplastic counterparts, NPD have the tendency to sorb chemicals from the ambient water (Zhang and Goss, 2020). Some studies, such as Norland et al. (2021) and Koelmans et al. (2022), indicated that the exposure route for microplastics associated chemicals is in most cases negligible due to the high levels of other particulate materials in the surrounding environment to which chemicals can sorb. Nevertheless, this may not necessarily apply to NPD (Mitrano et al., 2021). Because NPD have a smaller size compared to their microplastics counterparts, they may be able to carry the chemicals into organisms'

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bodies with a higher capacity compared to microplastics (Abdelsaleheen et al., 2021). Moreover, in terms of nanosafety, NPD might not increase the concentration of co-occurring chemicals in organisms' bodies in comparison to the surrounding environment, but they may carry the chemicals to a tissue or locations inside organisms' bodies that cannot be reached by the chemicals alone. For example, Trevisan et al. (2020) reported that PS-NPD increased the concentrations of the *co*-occurring polycyclic aromatic hydrocarbons (PAHs) in the yolk sac and the brain of zebrafish, while PAH-only exposure led to PAH accumulation in the yolk sac but not in the brain. Thus, even if the presence of NPD would play a negligible role in transferring chemicals into organisms (compared to other natural particulate materials), this does not rule out that NPD might change the toxicity profile of chemicals. This has been already documented in the literature. For example, some studies have reported that the presence of PS-NPD increased the toxicity of benzophenone-3 (Na et al., 2021) and silver ions (Abdolahpur Monikh et al., 2020) to *D. magna* as well as the toxicity of PAHs to zebrafish (Trevisan et al., 2020), while others reported that PS-NPD decrease the toxicity of PAHs to zebrafish (Trevisan et al., 2019). This contradiction in literature could be attributed to the differences in the types of chemicals, the physicochemical properties of the tested NPD, and the tested species. Systematic studies are required to fill the gap in knowledge and reveal how the simultaneous variation in the physicochemical properties of NPD influence their toxicity and the toxicity of their co-occurring chemical under chronic and acute conditions for environmental risk assessment.

The hydrophobic nature of NPD increases the sorption capacity of the particles for hydrophobic organic chemicals such as PAHs (Liu et al., 2016) Additionally, variation in different physicochemical properties of NPD, such as particle size, shape, chemical composition, degree of cross-linking and crystallinity (amorphous and crystalline) might play a significant role in the partitioning of the chemicals onto the particles and, consequently, in the toxicity of the chemicals (Kokalj et al., 2021). It has been documented that, for instance, the sorption of perfluorooctane sulphonate and perfluorooctanesulfonamide onto microplastics (PE and PVC) was higher than their sorption onto PS (Wang et al., 2015). Rochman et al. (2013) measured the sorption of polychlorinated biphenyls (PCBs) and PAHs over 1 year onto polyethylene terephthalate (PET), high-density polyethylene (HDPE), PVC, low-density PE (LDPE), and PP. They reported that the concentrations of PAHs and PCBs sorbed to HDPE, LDPE, and PP were significantly higher than those of PET and PVC. O'Connor et al. (2016) reviewed the literature and concluded that the sorption efficiency of chemicals to microplastics was as follows; Low-Density PE ≈ High-density PE ≥ PP > PVC ≈ PS. These results indicate clearly that the variation in the chemical composition of microplastics does influence their sorption capacity to chemicals, even if the particle size is equal. Although an increasing number of studies are reporting the influence of PS-NPD on the toxicity of their co-occurring chemicals (Lin et al., 2020; Singh et al., 2021; Bhagat et al., 2021), the open question is how the variation in the chemical composition (PP-NPD, PE-NPD and PVC-NPD) and particle size of NPD might modulate the toxicity of organic chemicals in organisms.

The objective of this study was to understand how the variation in the chemical composition and particle size of NPD alter their acute and chronic toxicity and the toxicity of their co-occurring organic chemicals in aquatic organisms. Our hypothesis is that the induced toxicity is different between NPD of the same particle size but different chemistries and between NPD of the same chemical composition but different sizes. Here, we were able to successfully prepare NPD of different sizes and chemical compositions, including PE (50 nm), PP (50 nm), PVC (200 nm) and PS (200 nm and 600 nm). Chronic effects of NPD and their co-occurring chemicals were investigated using an invertebrate, *D. magna*, and their acute effects were tested using zebrafish (*Danio rerio*) embryos. We selected Benzo(*a*)pyrene (BaP) as a model organic chemical. This chemical can potentially disrupt natural endocrinology of aquatic organisms (Sun et al., 2021). This chemical (log Kow = 6.04) has a high affinity to the surface of plastics (Lee et al., 2014) and it is commonly recognized as carcinogen and endocrine disruptor (Corrales et al., 2014). For example, oxidation of BaP can lead to the formation of reactive metabolites that can bind to DNA and cause reproductive toxicity in animals (Simão et al., 2021). Moreover, earlier work has indicated that BaP can induce malformation in zebrafish larvae (Huang et al., 2015).

2. Materials and methods

2.1. Materials

All chemicals and analytical grade solvents used in this study were purchased from Sigma Aldrich (Taufkirchen, Germany). Milli-Q water was supplied by a Millipore® filtration system (RiOs™ Essential 16 Water Purification System). Nanoparticles including PS 200 nm (PDI = 0.002), PS 600 nm (PDI = 0.05), PE 50 nm (PDI = 0.1), PP 50 nm (PDI = 0.1) and PVC 200 nm (PDI = 0.01) were purchased from CD-bioparticles (www.cd-bioparticles.net). The particles were stabilized using Tween 20. These particles were used as models of commonly observed plastics in the environment. We are aware that in the environment, the surfaces of particles undergo a transformation and natural organic matter can modify the surface of the particles (Wagner and Reemtsma, 2019). However, for simplicity, we performed this experiment with naked particles to reveal the direct effects of the particle chemistry on their toxicity. The culture medium for the *D. magna* (Elendt M7) was prepared freshly at the laboratory following the Organization for Economic Co-operation and Development (OECD) guideline Test No. 211 (OECD 211, 2012). All the glassware was washed with 10% nitric acid before use.

2.2. Preparing the stock dispersions and exposure media

Stock dispersions containing 1 g/L of NPD (PS, PP, PE, and PVC) were prepared in Milli-Q water and sonicated using a bath sonicator (35 kHz frequency, DT 255, Bandelin electronic, Sonorex digital, Berlin, Germany) for 5 min. Prior to the start of the experiments, the particles were diluted using a *D. magna* culture medium or zebrafish culture medium to reach the final particle number concentration of 3×10^{10} particles/L (equal to ~0.00025 mg of PE 50 nm, 0.00022 mg of PP 50 nm, 0.13 mg of PS 200 nm, 3.5 mg of PS 600 nm and 0.17 mg of PVC 200 nm) in the exposure media and then sonicated for 30 s. In this study, we used particle number as a dose metric to be able to compare the effect of particle size on their toxicity because particles of different sizes have different particle numbers in a unit mass (Abdolahpur Monikh et al., 2021). The number of the particles in the exposure medium was measured using dynamic light scattering (DLS, Zetasizer Nanodevice, Malvern Panalytical). The concentration of NPD in the environment is not available yet.

To produce BaP stock solutions, the chemical was dissolved in acetone (99.8%) to reach a final concentration of 1 g/L. The dissolution of BaP in acetone was diluted with Elendt M7 medium or zebrafish culture medium to reach the final concentration of 10 μg/L BaP and less than 0.3% of acetone. This concentration of BaP was selected based on some literature to have no lethal effects on the organisms (Atienzar et al., 1999: Della Torre et al., 2018: Sun et al., 2020: Simão et al., 2021). The concentration of the acetone in the exposure medium has neither toxicity effects on *D. rerio* embryos (Hallare et al., 2006) nor on *D. magna* (Leoni et al., 2008). The dissolution of BaP was used as a stock solution for different experiments. The amount of BaP was determined by using High-Performance Liquid Chromatography (HPLC-Agilent 1100 Series-diode array detector) (S1, Supporting Information). The BaP stock was transferred to sterile glass bottles and stored in the dark at 4 °C until used in toxicity studies.

2.3. Particle characterization

The hydrodynamic size of the particles was measured daily in triplicates using DLS, for 5 days in total. Determining the particle size over time allowed us to evaluate whether the particles underwent changes during the exposure because of agglomeration (Abdolahpur Monikh et al., 2019b). In this way, we confirmed that the organisms were exposed to single particles rather than agglomerates. The zeta potential of the NPD in the exposure media was measured using a Zetasizer Nanodevice (Malvern Panalytical, Netherlands). A transmission electron microscope (TEM, JEOL JEM-2100F) was used for imaging the NPD. The sample preparation for the TEM was performed following the previously published method (Abdolahpur Monikh et al., 2019b). Briefly, aliquots of the dispersion were diluted with MQ water and sonicated for 30 s. Immediately after sonication, the samples were put on copper grids and left to dry out for 24 h at room temperature. The grids were then used for TEM imaging.

To identify the polymer compositions, we used Raman spectroscopy. The highest spatial resolution for Raman spectroscopy is ~1 μm, which makes it unsuitable for identifying single NPDs. To circumvent this challenge, we prepared agglomerates of particles by drying droplets of the NPD (PP, PE, PS and PVC) dispersions on microscope slides for 24 h in darkness at room temperature. The samples were covered with Petri-dishes to avoid contamination due to air deposition. Raman spectra were measured with Thermo DXR2xi Raman microscope (Thermo Fischer Scientific). The laser wavelength was 785 nm and laser power 30 mW. Measurements were performed for all samples using a grating with 400 lines/mm producing spectral resolution 5 cm^{-1} and spectral range 3300–50 cm^{-1}, exposure time 0.33 s, and the number of scans 40. For larger agglomerates, the objective was 50×/0.75 NA and aperture 50 μm confocal pinhole, whereas for smaller agglomerates, the objective was 100×/0.90 NA and aperture 25 μm confocal pinhole, to achieve the highest spatial resolution limited by the laser wavelength. A background spectrum of the glass microscope slide was measured similarly from a clean spot on the slide. The polymer compositions of the NPDs were verified by comparing sample spectra to library spectra of plastic polymers. Before library identification, the spectrum of the PVC sample was corrected by subtracting the background spectra of glass. Other samples needed no corrections.

2.4. Maintaining the test organisms

The *D. magna* were fed three times per week with green algae (3×10^5 cells per L). All cultures were maintained at 20 °C at an 8:16 h light/dark photoperiod. The third brood produced by the original neonates was used for experiments. Juveniles were separated regularly from the cultural media.

Zebrafish (*D. rerio*) population originated from India and it was brought to Europe approximately 15 years ago. Zebrafish embryos were produced by adult females ($N = 12$) and males ($N = 24$; age of 200 days post-fertilization) kept in 320 L tanks in dechlorinated tap water (pH: 7.63 ± 0.15, nitrite <0.01 mg and temperature: 25.2 ± 0.28 °C and light/dark cycle 10:14 h). The spawners were placed in six three-liter spawning boxes (two females and four males each) overnight and in the subsequent morning embryos were collected and fertilized eggs were visually separated from the unfertilized ones.

2.5. Test design for reproduction toxicity in D. magna

A chronic experiment on *D. magna* was performed to analyze the effects of particle size and chemical composition on the life-history traits (survival and reproduction) of the organisms. A schematic illustration of the setup is provided in Fig. 1. Standard reproduction toxicity test was conducted in compliance with OECD guideline 211 (OECD 211, 2012). Third brood neonates (< 24 h) were used in this experiment. Each individual was put in a 60 mL uncovered glass jar filled with 50 mL of Elendt M7 medium ($n = 10$ animals for each treatment). The treatments were (a) control (no NPD and BaP), (b) BaP only, (c) NPD only and (d) NPD + BaP (Fig. 1).

The measured particle concentrations in the exposure media were between 2.7×10^{10} and 3.1×10^{10}. We selected this arbitrary concentration range based on two reasons. First, the concentration must be high enough to be detected in the medium by the available analytical techniques. Second, the concentration should be low enough to represent the environmental expected concentration of NPD, as close as the analytical techniques allow. The nominal particle number of the NPD was kept equal for all the treatments. Note that the particle number concentration might be dynamic, and the particle number concentration, thus, refers to the initial concentration.

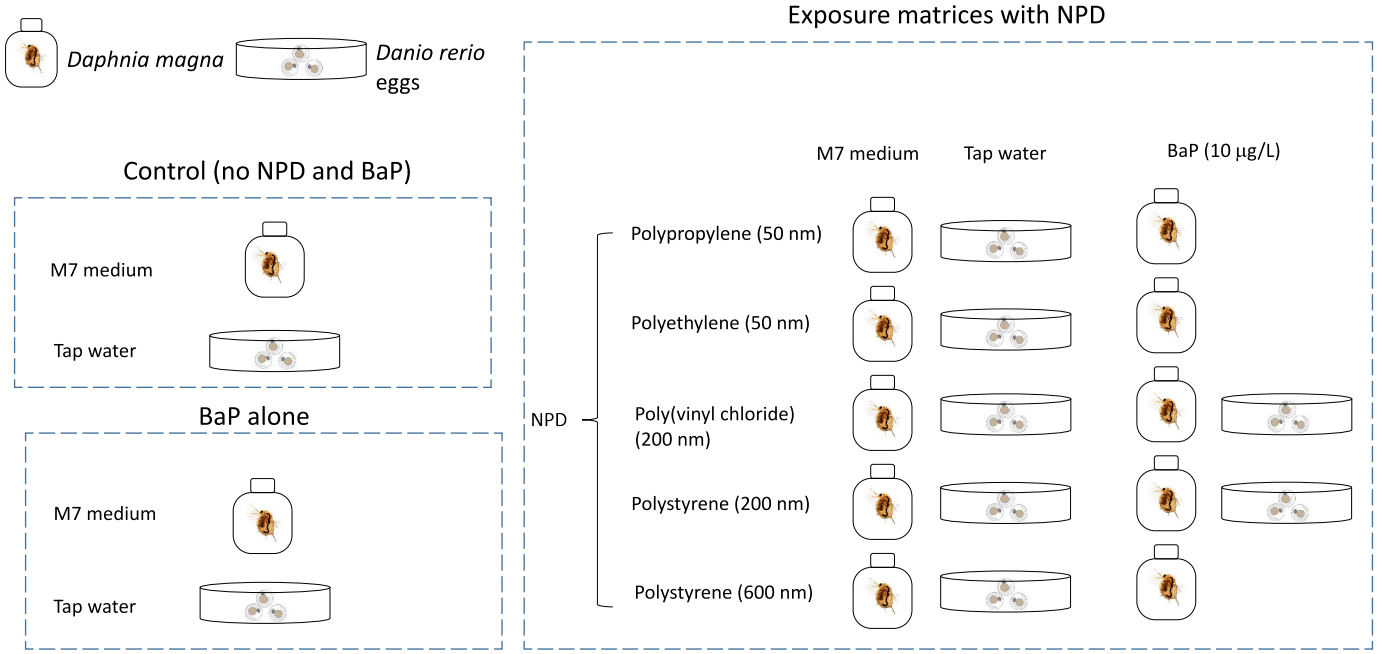

Fig. 1. A schematic illustration of the exposure matrices and the test design.

To prepare the mixture, BaP and NPD were mixed in dark at room temperature for 24 h prior to the exposure to increase the possible sorption of the BaP to the particles. We are aware that the partitioning of chemicals onto plastic and reaching an equilibrium depends on the type of plastics and might take more than 24 h (Hartmann et al., 2017). For simplicity, however, we mixed the particles and the chemicals only for 24 h regardless of whether the system reached an equilibrium or not. The exposure to BaP (10 μg/L) solution was also performed without the NPD (Fig. 1). This concentration of BaP was selected based on the earlier literature, demonstrating that the lethal concentration of BaP in *D. magna* is 0.25 ± 0.04 mg/L (Atienzar et al., 1999). In other words, we selected the concentration of BaP to be much lower than the lethal concentration to *D. magna*. In a separate experiment, the concentration of BaP in the medium without particles was also measured over time (S2, Supporting Information) to assure that the BaP did not sorb to the container surface and was distributed in the exposure media. Control samples, containing no substances were prepared in the same way using a clean Elendt M7 medium. The organisms were exposed for 21 days and their life-history traits (survival and reproduction) were recorded. The exposure media were renewed every 2 days and the feeding was carried out after each media renewal using green algae (3×10^5 cells per L). The organisms were gently transferred to a fresh medium by using a 5 mL plastic pipette to reduce handling stress. Survivors and immobile individuals were counted every day and the dead animals were removed. We checked the mortality following the OECD guidelines and the organism was considered dead when it was not able to swim, or if there was no observed movement of appendages or postabdomen, within 15 s after gentle agitation of the test container. The number of neonates, the mortality of the neonates and parents, the number of broods, and the delay in reproduction were recorded every day.

2.6. Test design for toxicity in zebrafish embryos

Fertilized zebrafish eggs, within 6 h post-fertilization (hpf), were used in this study. All embryos were derived from the same spawning trial but originated from different parents. Fertilized eggs were distributed in Petri-dishes (20 embryos per Petri-dish) with 20 mL of the medium used for culturing the parents (in tap water). The eggs were incubated in an environmentally controlled room (28 °C, 80% humidity) with an adjusted photoperiod (14 h light/10 h dark cycle). Each treatment had five replicates. Newly fertilized eggs were exposed to 3×10^{10} particles/L of PE 50 nm, PP 50 nm, PVC 200 nm, PS 200 nm and PS 600 nm (Fig. 1) in Petri-dishes. The measured concentrations were between 2.8×10^{10} and 2.9×10^{10} particles/L. This concentration of particles was selected based on the reasons mentioned above (see Section 2.5.). As illustrated in Fig. 1, the eggs were also exposed to BaP (10 μg/L); the mixture of PVC 200 nm + BaP (10 μg/L); and the mixture of PS 200 nm + BaP (10 μg/L). The concentration of BaP was selected based on a literature review to induce no lethal toxicity in zebrafish embryo (Weigt et al., 2011).

The exposure lasted for 24 h and the dead eggs were removed from the Petri-dishes every 12 h during the exposure. In the control, the eggs were not exposed to any substances. After 24 h, the eggs were transferred to Petri-dishes containing clean water without any substances. The embryonic/larval mortality and hatching rate (the ratio of hatched embryos to the remaining living embryos in each Petri-dish) were evaluated every 24 h for 120 hpf (before the larvae opened the mouth) in total and dead embryos were removed. Malformation of the larvae was noted at 120 hpf using a Wild M8 stereomicroscope (Wild Heerbrugg, Switzerland) and Dino-Lite digital camera. Treated and untreated larvae were euthanized with an overdose of tricaine methane sulfonate (Sigma Aldrich) before measuring for their standard lengths under a stereomicroscope.

2.7. Statistical methods

Statistical analyses were performed using the IBM SPSS Statistics 25 software. Prior to the analyses, the data were checked for normality using the Shapiro–Wilk tests. The effect of treatments on the number of neonates produced by daphnids, the number of hatched zebrafish embryos, and the length of zebrafish larvae between treatments were tested with One-Way Analysis of Variance (ANOVA) followed by Duncan post hoc tests. All data are reported as mean ± SD and differences between mean values (biologically independent samples) were deemed statistically significant if $p < 0.05$.

3. Results and discussion

3.1. Particle characterization

Before performing ecotoxicity tests, it is important to characterize NPD in terms of chemical composition, size, shape, and zeta potential because these physicochemical properties can influence the ecotoxicity of NPD (Abdelsaleheen et al., 2021). The chemical composition of the NPD is illustrated in Fig. 2. The polymers of the samples were characterized as PE, PP, PVC, and PS with Raman microspectroscopy and spectral library search. The physicochemical properties of the particles are summarized in Table 1 and the transmission electron microscope (TEM) images are illustrated in the Supporting Information (Fig. S2a). The TEM images showed that the particles were spherical in shape and no homoaggregation (aggregation between NPD of the same type) occurred between the particles. Some agglomerations were observable which could be the results of the drying step during the sample preparation for TEM (Abdolahpur Monikh et al., 2019a). The measured zeta potential for the particles in the zebrafish medium was highly negative (Table 1), indicating that the particles could be electrostatically stable against agglomeration in the medium due to particle-particle repulsions. This was confirmed by measuring the hydrodynamic size of the particle over time to determine the aggregation behaviour of the particles in each exposure media (Fig. S2b, Supporting Information). When dispersed in the daphnids exposure media (M7), the particles were still negatively charged, but the absolute value of the zeta potential slightly decreased (Table 1). This led to a slow agglomeration of the particles in the M7 medium over time (Fig. S2b, Supporting Information). The particles doubled in hydrodynamic size after 48 h in the M7 medium. Thus, the M7 exposure medium was changed every 48 h to expose the organisms to single particles rather than agglomerates.

3.2. Reproduction toxicity in D. magna

The effects on the reproduction of the daphnids were determined by counting the number of broods and the neonates produced by the exposed *D. magna* over 21 days. In all different treatments, the mortality of *D. magna* ranged from 10 to 30% (Fig. S3, Supporting Information). The mortality of 20% in the control groups was within the recommended limit by the OECD guidelines (OECD 211, 2012). The influence of NPD on the reproduction of daphnids can be observed over time in Table 2. Except for the daphnids exposed to PE 50 nm and PS 200 nm, all the daphnids produced 5 broods when exposed to NPD (Table 2). The total number of neonates produced in the first brood was lower than in the other four broods in all treatments, including the control samples. Although the number of neonates in broods 2 and 3 increased in all treatments, no significant differences were detected in the number of produced neonates between daphnids exposed to NPD and the controls.

The significant variation in the number of the produced neonates appeared in broods 4 and 5. The number of neonates in brood 4 of daphnids exposed to PE 50 nm and PS 200 nm reached the highest level compared to daphnids in other treatments, whereas, in brood 5 the number of the neonates decreased to zero in these two treatments. These findings suggest that, first, the reproduction toxicity in daphnids

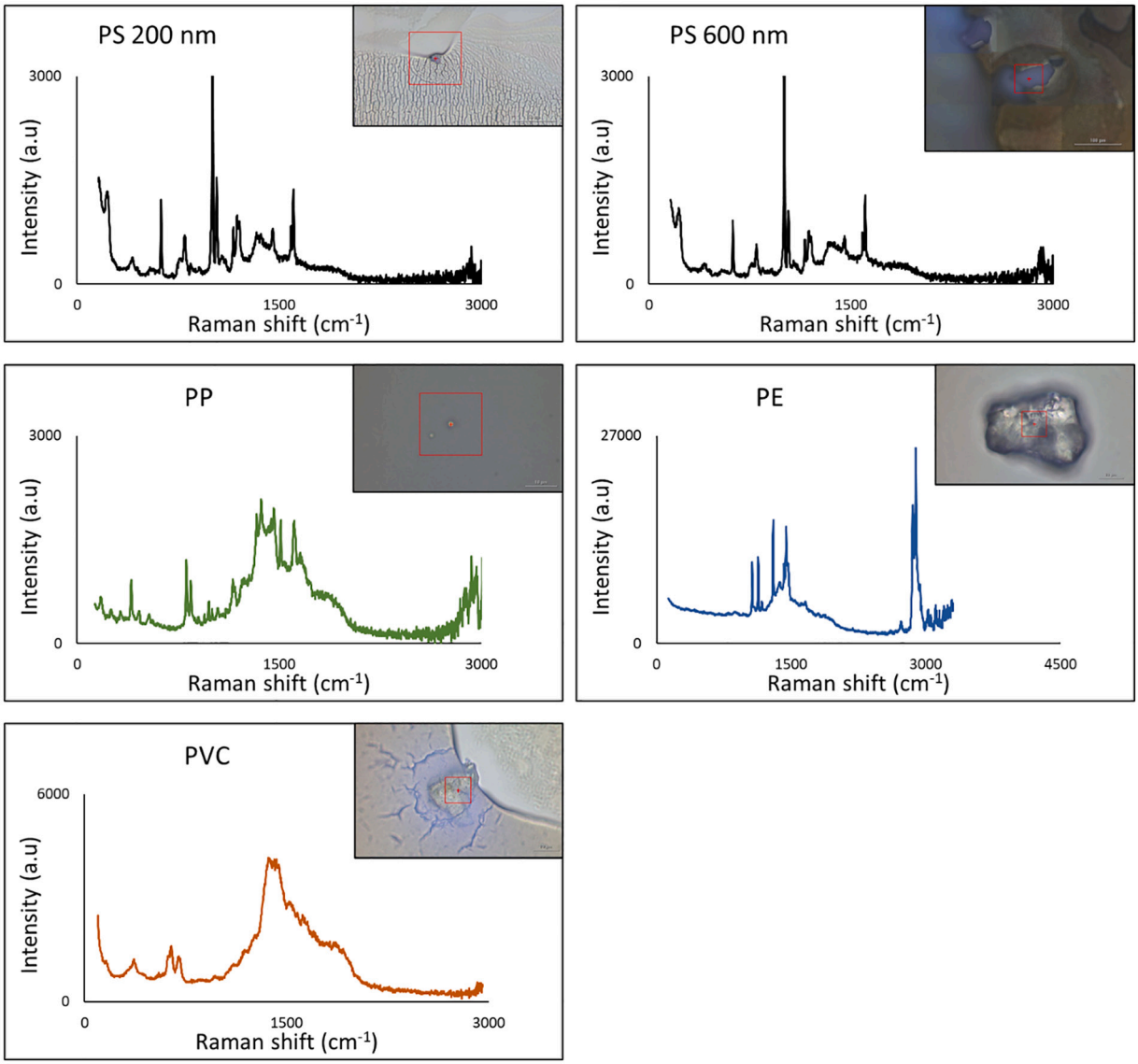

Fig. 2. Raman spectra and light microscope images of the NPD were used in this study. The uncorrected spectrum of PVC has also a broad peak at around 1500 cm-1 from the glass microscope slide.

Table 1
Physicochemical characterization of the NPD (mean ± standard deviation) used in this study.

NPD	Hydro- dynamic size	Transmission electron microscopy measured size	Zeta potential (mV)	
			Measured in zebrafish exposure media	Measured in daphnids exposure media
PE 50 nm	67 ± 15	50 ± 10	−23 ± 3	−16 ± 3
PP 50 nm	60 ± 12	53 ± 5	−25 ± 1	−19 ± 2
PS 200 nm	250 ± 36	207 ± 12	−23 ± 2	−17 ± 3
PS 600 nm	712 ± 54	650 ± 35	−22 ± 1	−19 ± 2
PVC 200 nm	291 ± 83	241 ± 28	−22 ± 2	−17 ± 3

exposed to NPD might appear only after long-term exposure, indicating that chronic ecotoxicity tests are required to reveal the sub-lethal adverse effects of NPD. Second, the size of NPD, when the chemical composition is the same (PS 200 versus PS 600 nm), and the chemical composition, when the size is the same (PS 200 versus PVC 200 and PE 50 versus PP 50), alter the potency of NPD toxicity on reproduction in *D. magna*. Previous studies have shown that exposure to 0.1 mg/L and 50 mg/L PS NPD significantly decreased the number of neonates in *D. pulex* (Liu et al., 2019) and *D. galeata* (Cui et al., 2017), respectively. Rist et al. (2017) exposed *D. magna* to 2 μm and 100 nm PS particles (1 mg/L) for 24 h, followed by a 24 h depuration period in a clean medium. No influence on the reproduction was found in 21 days experiment despite the high body burdens of both particles. Similarly, Kelpsiene et al. (2020) demonstrated that after 103 days of exposure to PS NPD, the total reproductive output of *D. magna* was not significantly affected. However, there was a decreasing trend in the number of neonates over their lifetime. The current study documents for the first time that NPD other than PS also can induce reproduction toxicity in *D. magna* after 21 days of exposure. The main variation between the testing design of our study and that in previous studies, which could to a high extent explain

Table 2
The number (mean ± standard deviation) of neonates per brood produced by *D. magna* in each treatment.

	Without BaP					With BaP				
	B1	B2	B3	B4	B5	B1	B2	B3	B4	B5
Control	15 ± 2	45 ± 11	57 ± 19	60 ± 8^{a}	53 ± 13^{a}	16 ± 3	42 ± 4	54 ± 7^{a}	63 ± 6^{cd}	58 ± 13
BaP only	–	–	–	–	–	17 ± 3	45 ± 8	54 ± 5^{a}	66 ± 5^{cd}	61 ± 9
PP 50 nm	17 ± 4	47 ± 10	62 ± 8	66 ± 10^{ab}	67 ± 16^{b}	17 ± 2	42 ± 4	55 ± 5^{a}	61 ± 5^{c}	57 ± 10
PE 50 nm	18 ± 4	49 ± 9	57 ± 8	67 ± 5^{ab}	0	21 ± 5	63 ± 7	67 ± 8^{bc}	67 ± 6^{cd}	0
PVC 200 nm	18 ± 2	50 ± 4	93 ± 9	58 ± 17^{a}	68 ± 9^{b}	18 ± 5	62 ± 8	62 ± 7^{b}	69 ± 5^{d}	62 ± 9
PS 200 nm	20 ± 8	55 ± 14	67 ± 7	72 ± 9^{b}	0	17 ± 3	60 ± 6	69 ± 9^{c}	47 ± 15^{a}	0
PS 600 nm	19 ± 3	49 ± 11	67 ± 6	60 ± 6^{a}	76 ± 7^{c}	16 ± 4	57 ± 3	68 ± 6^{bc}	40 ± 12^{a}	0

a,b,c letters show the significant differences (ANOVA, $p < 0.05$) between the number of neonates produced by daphnids of different treatments. The results are the average of 10 samples. Degree of freedom is 9. B: Brood.

the variation in the findings, is that we performed the tests by using particle number as a dose metric whereas previous studies used mass. Particle number is the recommended dose metric for toxicological testing of particulate materials when the influence of particle size and shape are the subject of the studies (Abdolahpur Monikh et al., 2021). Using this systematic design, we here show that the size and composition of NPD do influence their toxicological potency on the reproduction of *D. magna*.

One question that arises in light of the present findings is the increase in the number of neonates in the fourth brood of *D. magna* exposed to PE 50 nm and PS 200 before reaching zero neonates in the fifth brood. It is likely that the organisms already spent a high amount of energy to produce the fourth brood which minimized the available energy to produce the fifth brood. Similar results have been reported for other toxicants, where exposure to metals altered the physiological energy in daphnids and minimized the available energy for reproduction (Knops et al., 2001). This pattern clearly highlights the importance of understanding the NPD-type-mediated trade-off processes in regulating the distribution of physiological resources and energy between reproduction and other life-historical activities.

When *D. magna* were exposed to the mixtures of NPD + BaP, there was a more pronounced association between the physicochemical properties of the NPD (size and chemical composition) and the induced reproduction toxicity. The mortality in daphnids due to the exposure to BaP alone was 10% with no significant difference compared to the control ($p < 0.05$). The BaP alone did not induce any reproduction toxicity in the daphnids when compared to the control (Table 2). Similar to the daphnids exposed to NPD alone, the number of neonates in the first brood of daphnids exposed to the mixture of NPD + BaP was lower than that in the other broods (Table 2).

Unlike the exposure to NPD alone, the exposure to the mixture of NPDs + BaP induced an earlier alteration in the number of produced neonates (appeared in the brood 3) between the treatments (Table 2). The number of neonates of brood 3 in PS 200 nm + BaP treatment was significantly higher than in the control. This was followed by the daphnids exposed to the mixture of PE 50 nm + BaP and PS 600 nm + BaP. In brood 4, the number of neonates in the daphnids exposed to the mixture of PS 200 nm + BaP and PS 600 nm + BaP decreased significantly compared to the control and other treatments. No neonates were produced in brood 5 due to the exposure to PE + 50 nm, PS 200 nm + BaP and PS 600 nm + BaP. This finding suggests that the mixture of BaP with PS NPD could have a higher influence on the reproduction of *D. magna* than the mixture of BaP with other types of NPD (i.e. PE, PP, and PVC). Previous studies have reported different results for the combined adverse effects of PS + PAHs. For example, Lin et al. (2020) modeled the combined effects of PS NPD and PAHs to *D. magna*. They reported that the PS NPD matrix would retard the uptake of PAHs in *D. magna*, especially for the less hydrophobic PAHs. They used 100 nm PS NPD and exposed the organisms for up to 36 h, which could explain the differences between their findings and our results. Regarding other organic chemicals, Lin et al. (2019) reported that PS particles could decrease the toxicity of polychlorinated biphenyls to *D. magna*. We are not aware of any study that would have focused on understanding the combined effects of NPD with different chemical compositions (e.g., PP, PE and PVC) and PAHs on organisms. Some studies are available for microplastics, showing that the chemistry of microplastics may influence the toxicity of some organic substances. For example, Zocchi and Sommaruga (2019) investigated the combined effect of glyphosate (glyphosate acid, glyphosate-monoisopropylamine salt, and Roundup Gran) and microplastics (PE and PET/polyamide fibers) on *D. magna*. They reported that the variation in the toxicity of glyphosate formulations caused by microplastics is attributed to the differences in the sorption of the glyphosate-based formulations to the microplastics. Our finding shows that in addition to the direct influence of the particle chemical composition and size, these physicochemical properties can alter the toxicity of organic chemicals, such as PAHs.

3.3. Toxicity to zebrafish embryo

3.3.1. Hatching rate

There was no significant mortality in the embryos exposed to NPD in comparison to the control (Fig. S4, Supporting Information). Hatching of the embryos started at 24 hpf with no significant differences between the treatments (Table 3). After 48 hpf, the highest number of hatched embryos was recorded for the control (8 ± 0.4 embryos) followed by those exposed to PVC 200 nm (7 ± 0.4 embryos). The lowest number of hatched embryos was observed in the group exposed to PS 200 nm (1 ± 1.0 embryos). Interestingly, most of the embryos exposed to PS 200 nm hatched after 72 hpf, indicating a delay in the hatching time compared to the control. The same delay in hatching was observed also for the group exposed to PP 50 nm, PE 50 nm, and PS 600 nm. This is of paramount importance for risk assessment of plastics because delay or inhibition of hatching, as a sub-lethal effect, can cause life-threatening exposure to the species. These results are in agreement with a

Table 3
The number (mean ± standard deviation) of hatched zebrafish embryos per day after exposure to NPD.

	Number of exposed eggs	24 hpf	48 hpf	72 hpf	96 hpf
Control	20 20	3 ± 1	8 ± 0.4^{d}	8 ± 0.5^{b}	0
PP 50 nm	20	2 ± 0.8	6 ± 1^{c}	9 ± 0.3^{b}	0
PE 50 nm	20	2 ± 0.5	4 ± 1^{b}	10 ± 2^{bc}	0
PVC 200 nm	20	2 ± 1.5	7 ± 0.4^{cd}	4 ± 1^{a}	0
PS 200 nm	20	2 ± 1.5	1 ± 1^{a}	10 ± 1^{bc}	0
PS 600 nm	20	2 ± 0.5	4 ± .37^{b}	9 ± 2^{b}	0

Twenty eggs were exposed in each treatment.
a,b,c,d letters show the significant differences (ANOVA, $p < 0.05$) between the number of hatched embryo in different treatments.

previous study that showed that PS NPD could change the mechanical properties of chorion and induce inhibition of zebrafish embryos hatching at 72 hpf (Duan et al., 2020).

By decreasing the particle size of PS NPD from 600 nm to 200 nm, the hatching success decreased significantly, suggesting size-dependent adverse effects of NPD. This is in agreement with a previous study, showing that smaller NPD can cause a more severe hatching inhibition in zebrafish (Lee et al., 2019). One explanation could be the possible penetration of the 200 nm NPDs through the chorionic pores, which have a diameter of up to 600 nm (Chen et al., 2020). Particles with size ≥600 nm are unlikely to penetrate the chorionic pores as were reported previously for PS NPD (van Pomeren et al., 2017) and other nanomaterials (Chen et al., 2020). But the question that may arise is why the PVC 200 nm did not show similar toxicity as the PS 200 showed, despite having the same size. The reason for this finding might be searched in the potency of the PVC in inducing hatching inhibition in zebrafish embryos, which could be lower than the potency of PS. This highlights the importance of performing systematic studies to simultaneously understand how specific properties of NPD can alter the possible adverse effects of the particles while all other physicochemical properties are kept the same. For example, our findings documented the various potencies of NPD of different chemical compositions to induce hatching inhibition in zebrafish embryos, even if the size of the particles are equal (PVC 200 nm versus PS 200 nm and PE 50 nm versus PP 50 nm). Such results can reduce the conflicting observations because debate persists regarding the size-dependent uptake (Booth et al., 2016) and adverse effects of NPD, as reported for other nanomaterials (Chen et al., 2020). The combination of physicochemical properties such as size, shape, and composition simultaneously influence the uptake and adverse effects of NPD and one cannot extrapolate results obtained for PS NPD to other types of NPD, despite having the same size.

We also investigated the effect of NPD of different chemical compositions, but the same size, (PVC 200 nm and PS 200 nm) on the toxicity of BaP to the zebrafish eggs. The mortality of embryos upon treatment with the mixture of NPD + BaP was lower than 20% and similar to the control (Fig. S4, Supporting Information). The adverse effects of BaP on the eggs without NPD were also tested (Table 4). Unlike the control (Table 3), most of the embryos in the BaP treatment hatched after 72 hpf and the hatching continued at 120 hpf. The negative influence of BaP on the hatching of zebrafish embryos was expected and has been previously documented (Huang et al., 2015). When exposed to the mixture of NPD and BaP, the number of hatched embryos increased significantly after 24 hpf compared to the BaP alone (Table 4). Most of the embryos hatched after 48 hpf in the treatment containing the mixture of NPD + BaP, unlike the BaP alone (after 72 hpf). This indicates that the presence of NPD could decrease the adverse effects of BaP on the hatching of the embryos. One possible explanation is the sorption of BaP to the particles and decrease in the bioavailability of the BaP to the zebrafish embryo, as was reported for other PAHs (Yu et al., 2019).

Although both PVC and PS NPD have eliminated the effect of BaP on the hatching inhibition of zebrafish embryos, however, there was a difference between how the two NPD reduced this effect. In the treatment containing the PS 200 + BaP, some embryos hatched at 96 hpf, similar to the BaP alone. Whereas, in the treatment containing the PVC 200 + BaP no hatching was observed at 96 hpf, which was similar to the PVC 200 nm alone (Table 3). This finding contradicts the previous study, which reported that PS NPD could enhance the toxicity of PAH, e.g., phenanthrene (Zhang and Goss, 2020), and cause delays in the hatching of zebrafish embryos. The combined effects of one type of NPD with different types of PAHs (i.e., BaP and phenanthrene) are distinct. The difference in the elimination of the BaP effects on hatching by PVC 200 nm and PS 200 nm might be related to the affinity of BaP to sorb onto the two particles. For example, Lee et al. (2014) performed an experiment on the sorption of PAHs onto PE, PS, and PVC. Their results show that the sorption of PAHs varies between different plastic debris and hydrophobicity was the main interaction mechanism for the sorption behaviour between PAHs and the particles.

Table 4
The number (mean ± standard deviation) of hatched zebrafish embryos per day after exposure to the mixture of NPDs and BaP.

	Number of exposed eggs	24 hpf	48 hpf	72 hpf	96 hpf
BaP	20	2 ± 1.5[a]	3 ± 0.8[a]	12 ± 1.7[b]	1 ± 0.5[b]
PVC 200 nm + BaP	20	3 ± 1.8[b]	7 ± 0.7[b]	7 ± 1[a]	0[a]
PS 200 nm + BaP	20	3 ± 1.3[b]	8 ± 0.8[b]	7 ± 1.2[a]	1 ± 0.5[b]

Twenty eggs were exposed in each treatment.
a,b letters show the significant differences (ANOVA, $p < 0.05$) between the number of hatched embryo in different treatments.

3.3.2. Larvae length and deformation

After hatching, we evaluated the influence of NPD and the mixture of NPD + BaP on the length and morphology of the hatched larvae (120 hpf). We did not observe any obvious morphological differences such as heart edema, axial curvature, head deformities between the control and any of the treatments, except for the larvae hatched from eggs exposed to PP 50 nm. As shown in Fig. 3a, exposure of eggs to PP 50 nm caused embryos to fail to develop the normal morphology and lead to spine curvature malformation in 18% of the larvae (Fig. 3a-b) which was significantly higher than the control (3%). This malformation has been reported previously as a typical malformation of zebrafish embryos upon exposure to nanomaterials (Duan et al., 2013). Such malformations are not due to any initially present contaminants, as the larvae in the control did not show a considerable number of malformation (only 3%) (Fig. 3b). There was also no difference between the percentage of malformed larvae in the control and in the BaP treatment (Fig. 3b).

Control larvae grew to 3.99 ± 0.1 mm at 120 hpf, significantly ($p < 0.05$, Fig. 3c) larger than in other treatments. The effects of NPD on the length of larvae were influenced by the size and chemical composition of the particles. Larvae of the PE 50 nm and PP 50 nm treatment had the smallest size followed by larvae of the PS 200 = PVC 200 nm > PS 600 nm treatments. These results suggest that particles with a smaller size could have the highest effects on the length of zebrafish larvae. One explanation is that due to their smaller size, PE and PP could penetrate the eggs and the embryos, consequently influencing the developmental stage of embryos and larvae as reported previously (Zhang and Goss, 2020).

4. Conclusions

Our results demonstrate that the toxicity potency of NPD is influenced by their chemical compositions and size. Against the common belief assuming that particles with smaller sizes can induce more adverse effects, we found that the combination of chemical composition and size together induce the adverse effects. For example, we found that the effect of 200 nm PS-NPD on the reproduction of *D. magna* is higher than the effect of 50 nm PP-NPD. We also showed that the toxicological effects of PE 50 nm and PP 50 nm are different despite having the same size, and the toxicological potency of PS 200 nm and PS 600 nm was also different despite having identical chemical compositions. In natural surface waters, NPD may be covered by natural organic matter. Future studies are needed to understand how the presence of natural organic matter can modulate the toxicity of the particles to aquatic organisms. The physicochemical properties of NPD influence also the toxicity of co-occurring BaP. In the presence of BaP, NPD showed an earlier influence on the reproduction of the daphnids compared to NPD alone. However, the presence of NPD could decrease the adverse effects of BaP on the hatching of the zebrafish embryos. Given the ubiquity of NPD of different sizes, shapes, and chemistries in aquatic systems, we expect

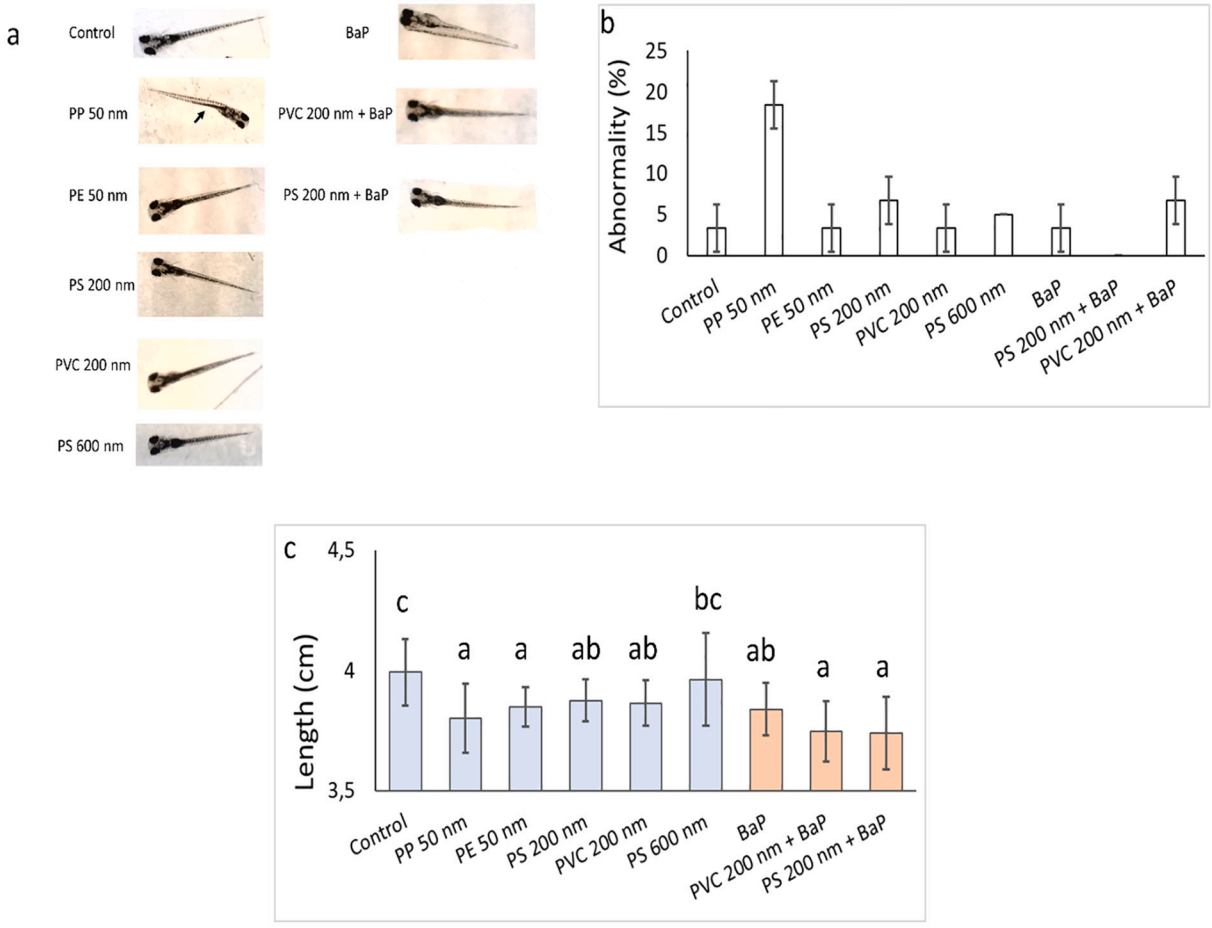

Fig. 3. Development of zebrafish larvae after 96 hpf. a) Observed malformation of the larvae (120 hpf) in PP 50 treatments compared to larvae from other treatments. b) The percentage of tail malformation in all treatments. c) The measured standard length of larvae at 120 hpf from treatments with and without BaP. Data are presented as mean ± S.D. ($n = 10$). The letters (a, b, c and d) show the significant differences between the treatments at $p < 0.05$ as assessed by one-way ANOVA followed by Duncan post-hoc tests.

that the risk assessment of these contaminants will face a challenge. It is likely that different types of NPD may induce different toxicities with different potencies. This may also complicate developing strategies such as read-across and grouping for NPD.

We recommend the future studies to comprehensively test the combined effects of physicochemical properties, such as size, shape, and composition simultaneously on the adverse effects of NPD rather than a single property. When we compare the results observed for *Daphnia magna* and *Danio rerio*, it can be observed that the adverse effects of the same NPD + PAHs mixture on various aquatic organisms are also very different, which indicates that the results cannot be extrapolated to different aquatic organisms.

C.A. Statement

F.A.M. designed the experiments, conceptualized, supervised, wrote and reviewed the study. F.A.M., M.D., and P.V·K performed the OECD daphnids reproduction test. F.A.M. prepared the dispersion of the particles and characterized the particles. F.A.M. and S·U-H maintained the zebrafish. F.A.M, H·H, R.K., J.K and S·U-H performed the fish exposer to NPD and toxicity tests. E.U. performed the Raman measurements. R.K, H·H, J.A and J.K contributed to supervising, writing, and editing the paper.

Declaration of Competing Interest

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

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