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MENG, Zijiao, et al.

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Acute toxicity of nanoplastics on Daphnia and Gammarus neonates: Effects of surface charge, heteroaggregation, and water properties

Zijiao Meng a, Rémi Recoura-Massaquant b, Arnaud Chaumot b, Serge Stoll a,⁎, Wei Liu a,⁎

a Department F.-A. Forel for Environmental and Aquatic Sciences, University of Geneva, Carl-Vogt 66, CH-1211 Geneva, Switzerland
b INRAE, UR RiverLy, Laboratoire d’écotoxicologie, F-69625 Villeurbanne, France

HIGHLIGHTS

• Stability of PS-NPLs is related to the freshwater properties.
• Toxicity of PS-NPLs is size-dependent and exposure condition-dependent.
• PS-NPLs are 1.3–6.5 times more toxic in mineral water compared to lake water.
• Surface charge and aggregation behavior are the most influential ecotoxic drivers.

ABSTRACT

Despite progress in evaluation of risk assessment, knowledge gaps largely exist understanding the toxicity of nanoplastics in aquatic systems considering nanoplastics surface properties, environmental media characteristics and species ecological traits. In this study, amidine - functionalized polystyrene nanoparticles (PS-NPLs) of 20, 40, 60 and 100 nm are considered using Geneva lake water and mineral water to investigate the behavior and effects in neonate organisms of the plankton Daphnia magna and the benthos Gammarus fossarum. Key parameters including ζ-potential, z-average diameter, conductivity, polydispersity index, pH, EC50 were investigated. The results showed that the toxicity of PS-NPLs exhibited a dose-response relationship, size- and exposure condition-dependent trend. The smaller size PS-NPLs (20 and 40 nm) induced higher adverse effects than PS-NPLs of 60 and 100 nm in both water conditions and crustacean species. Moreover, PS-NPLs were found more toxic in the mineral water compared to lake water. Principal component analysis evidenced that the surface charge and aggregation behavior are the most influential toxicity of PS-NPL factor for D. magna and Gammarus fossarum, respectively. These results highlight the relationship between PS-NPLs intrinsic properties, their transformation behavior, water properties and species-specificity in the evaluation of PS-NPLs biological effects on crustacean neonates in natural aquatic environments.

1. Introduction

By 2050, it is expected that 12 billion tons of used plastic will directly go to landfill or be discarded (Geyer et al., 2017). Once in the environment plastic waste will be transformed into microplastic and nanoplastics (NPLs) via physical, chemical, and biological degradation processes (Boucher and Priot, 2017; El Hadri et al., 2020; Shen et al., 2019). Microplastic contamination in food and fresh waters, such as table salt (Gündoğdu, 2018; Iñiguez et al., 2017; Yang and Nowack, 2020), drinking water treatment plants (Kirstein et al., 2021; Koelmans et al., 2019; Li et al., 2020; Negrete Velasco et al., 2022) have been systematically documented and investigated. Microplastic pollution is ubiquitous: from high-mountain lakes to deep-sea sediments (Esposito et al., 2022; Pastorino et al., 2022; Woodall et al.,...
2020), while nanoplastics as further degradation products of ageing microplastics (Enfrin et al., 2020; Gonzalez-Pleiter et al., 2019; Li et al., 2020) have larger surface-to-volume ratio and higher surface reactivity. Even more concerning is that nanoplastics are probably widely distributed and more toxic than microplastics (Gaylarde et al., 2020; Hazeem et al., 2020; Khoshnamvand et al., 2021; Yan et al., 2021). Based on the well-accepted fact that freshwater compartments have become major sinks for plastic pollutants (Boyle and Ormecci, 2020; Pochelon et al., 2021), it is necessary to improve a comprehensive understanding on the behavior of nanoplastics in freshwaters and their impact on freshwater organisms.

Micro and nano plastic detection and characterization in aquatic systems are predominantly investigated based on their size, shape, color, polymer type, and their interaction with water components (Ileavia, 2021; Mattsson et al., 2018). Based on current data on the occurrence of microplastics in the global aquatic environment, polystyrene is one of the most frequently detected and reported polymers (Coyle et al., 2020; Koelmans et al., 2019). It is mainly derived from personal care products and detergents (Elkhatib and Oyandel-Craver, 2020) and also the most frequently laboratory-studied NPLs particle involved in ecological hazard assessment of freshwater compartments (Allimi et al., 2022; Yang and Nowack, 2020). Polystyrene nanoplastics (PS-NPLs) with single-surface functional group have about 95% passive adsorption surface and carry stable positive (or negative) charges over a wide pH range (Pochelon et al., 2021). The presence of organic or inorganic particles can significantly affect the stability of PS-NPLs (Pradel et al., 2021; Singh et al., 2019). During the formation of hetero aggregates of PS-NPLs, natural organic matter (NOM), such as alginate, has more optimal aggregation-inducing effect than inorganic colloids (FeO(OH) and relative concentrations can both play a controlling role (Oriekhova and Stoll, 2018). The salt type (CaCl₂ or MgCl₂) and ionic strength of medium has also a significant effect on the stability of PS-NPLs (Shams et al., 2020). The surface charge and aggregation state of positively charged PS-NPLs can be more significantly modified by NOM (e.g., alginates or Suwannee River humic acid, SRHA) than negatively charged PS-NPLs (Saavedra et al., 2019). Those results indicate that the stability and surface properties of NPLs in the aquatic environment depend on their intrinsic physicochemical properties (size, surface charge, etc.) and extrinsic environmental variables (medium component such as ionic strength and pH). However, there is a significant knowledge gap with respect to the contribution of these impact factors and the behavior of NPLs in natural freshwaters.

Indeed, beside the different stability behavior of NPLs under the exposure conditions, few studies focus on the biological effects of NPLs in aquatic environment. Previous studies showed that compared to 1 μm PS particle, 100 nm PS-NPLs caused the photosynthetic parameters (e.g., photosynthesis activity) to change to achieve pH values in the range of 4–10 by adding NaOH and HCI (0.05 M). The pH was adjusted to 3.0 using 0.05 M HCl to keep the system stable. All stock solution of PS-NPLs were stored in the dark at 4 ºC. Evian bottled mineral water was bought from a local supermarket. The Evian water were left open to stand for 24 h before use. Geneva lake water was collected from lake of Geneva and was then filtered through a hydrophilic polycarbonate membrane 0.2 μm filter (GTTP04700, Merck Millipore Ltd., Switzerland). All freshwater samples were stored at 4 ºC and protected from light. The major water components (major ions) and properties (conductivity, pH) of Evian water and Geneva lake water are listed in supporting information (Tables S1 and S2).

2. Materials and methods

2.1. Preparation of PS-NPLs and Freshwater conditions

Surfactant-free aqueous suspension (4 % w/v) of amidine functionalized polystyrene (PS-NH₂) nanospheres with primary sizes of 20, 40, 60 and 100 nm were purchased from Invitrogen (Thermofisher science, Waltham, MA, USA). 1000 mg L⁻¹ of PS-NPLs were prepared by diluting of 40 g L⁻¹ stock solution in ultrapure water. The pH was adjusted to 3.0 using 0.05 M HCl to keep the system stable. All stock solution of PS-NPLs were stored in the dark at 4 ºC. Evian bottled mineral water was bought from a local supermarket. The Evian water were left open to stand for 24 h before use. Geneva lake water was collected from lake of Geneva and was then filtered through a hydrophilic polycarbonate membrane 0.2 μm filter (GTTP04700, Merck Millipore Ltd., Switzerland). All freshwater samples were stored at 4 ºC and protected from light. The major water components (major ions) and properties (conductivity, pH) of Evian water and Geneva lake water are listed in supporting information (Tables S1 and S2).

2.2. ζ-Potential and z-average hydrodynamic diameter of NPLs as a function of pH in ultrapure water

PS-NPLs suspensions at 20 mg L⁻¹ were prepared by diluting 1.6 mL of stock solution in 78.4 mL of ultrapure water. The pH of PS-NPLs was changed to achieve pH values in the range of 4–10 by adding NaOH and HCl (0.05 M). The pH was measured with a pH meter (Mettler Toledo, Columbus, USA). ζ-Potential and z-average hydrodynamic diameter of PS-NPLs at different pH values were determined with the mean of five replicates by using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd.: Malvern, UK) at 25 ºC.
2.3. \(\zeta\)-Potential and z-average hydrodynamic diameter of NPLs in natural lake water and mineral water

Four PS-NPLs were respectively dispersed in natural lake water (pH 8.05 ± 0.10, conductivity 288 ± 1 \(\mu\)S/cm) and mineral water (pH 7.2 ± 0.1, conductivity 575 ± 1 \(\mu\)S/cm), and then prepared into a series of 20 mg L\(^{-1}\) homogeneous solutions. \(\zeta\)-Potential and z-average hydrodynamic diameter were measured at 25 °C by Zetasizer Nano ZS at 0 h, 0.5, 1, 2, 4, 8, 24, 48 h. All samples were loaded through disposable folded capillary cells. Each sample was measured 5 times, each measurement containing 10 runs.

2.4. Scanning Electron Microscopy analysis

The morphology of PS-NPLs in different media was observed by using SEM (JEOL JSM-7001FA, Tokyo, Japan). The samples were carried out in aluminum holders covered with 5 \(\times\) 5 mm silica mirrors (Agar Scientific (G3390)). PS-NPLs of 20 and 100 nm were dispersed in ultrapure water, natural lake water, and mineral water to prepare 10 mg L\(^{-1}\) homogeneous working solutions. After standing for 24 h, the measurement samples were prepared by taking 5 \(\mu\)L of the suspension, naturally dried on the holders, and wrapped with a 5 nm gold coating.

2.5. Bioassays

2.5.1. D. magna

The acute toxicity of PS-NPLs of different primary sizes in natural lake water and mineral water was assessed using a Daphtoxkit F test. It uses uniform-sized, same-age D. magna neonates hatched from ephippia, which provides similar sensitivity to conventional toxicity testing using neonates from stock cultures (Microbiotests, 2019). Ephippia was incubated for 72 h in 50 mL pre-aerated standard fresh water (\(\text{NaHCO}_3\) [64.75 mg L\(^{-1}\)]; \(\text{CaCl}_2\cdot2\text{H}_2\text{O}\) [294 mg L\(^{-1}\)]; \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\) [123.25 mg L\(^{-1}\)]; \(\text{KCl}\) [5.75 mg L\(^{-1}\)]) at constant temperature 21 °C and continuous light at 6000 lx. Neonates were pre-fed with 2 mL \(R.\ subcapitata\) (5 \(\times\) \(10^5\) cells mL\(^{-1}\)). Then at 2 h before the toxicity test, neonates were transferred to multi-well test plates containing 10 mL of test solution per well, 5 neonates per well. Each test solution and blank control were repeated 4 times.

2.5.2. G. fossarum

Adult G. fossarum were collected, through 2- and 2.5-mm sieves, on a pristine site (old watercress farm - St Maurice de Rémens, Ain, FRANCE) and quickly transported to the Ecotoxicology laboratory in INRAE Lyon with ambient freshwater. During laboratory acclimatization, the organisms were maintained for 1 week in 20-L tanks under constant aeration and continuously supplied with drilled groundwater, a 16/8-h light/dark photoperiod, and a temperature of 12 °C ± 0.5 °C. One day before the exposure, one hundred of gammarid pairs with females bearing embryos at embryonic development stage 5 and close to molting (Geffard et al., 2010) were isolated in order to synchronize the release of neonate juveniles. After 24 h, released neonates were transferred to multi-well test plates containing 10 mL of test solution per well, 5 neonates per well. The organisms were exposed to PS-NPLs of different primary sizes with increasing concentrations in the range of 0.5–20 mg L\(^{-1}\) in natural lake water and mineral water. The multi-well test plates were incubated at...
constant temperature of 20 °C for 24 h in the dark. Mortality rates were calculated by averaging the number of dead and actively swimming neonates in each well. The light microscopy (OLYMPUS, U-TV1X-2, T2 Tokyo, Japan) equipped with a digital camera is used for the observation of organisms.

2.6. Statistics analysis

The data normality was checked using Chen-Shapiro test and Shapiro-Wilk test. The visualization of PS-NPLs behavioral data was realized by “Graph” function in OriginLab 8.5 (OriginLab Corporation, USA). The ecotoxicity (EC50 value) and dose-response curves of PS-NPLs with different primary sizes in different freshwater conditions were determined by “DoseResp” model. The correlations and principal component analysis (PCA) among behavioral characteristics, environmental variables and associated ecotoxicity were implemented with “FactoMineR”, “PerformanceAnalytics” package in R version 4.0.3.

3. Results and discussion

3.1. Influence of pH on the ζ-potential and z-average hydrodynamic diameter of NPLs in ultrapure water

Before observing the behavior of PS-NPLs with different primary sizes in different freshwater conditions, the ζ-potential and z-average hydrodynamic diameter of PS-NPLs were firstly examined in ultrapure water at a concentration of 20 mg L⁻¹ and by adjusting the pH from 4.0 to 10 (Fig. 1 A–D). In this pH range, ζ-potentials of all tested PS-NPLs exhibit positive values in ultrapure water and decrease steadily by increasing the pH, ranging from +65.8 ± 1.9 mV to +20.6 ± 0.7 mV. This indicates that all tested PS-NPLs in ultrapure water are positively charged in a wide pH range. A limited amount of surface charges is neutralized by pH increase due to the high pKa value of amidine functional group, which is similar to the previous reported studies (Oszczapowicz and Raczyńska, 1984; Ramirez et al., 2019). Moreover, their z-average hydrodynamic diameters remain essentially stable in this pH range. Compared with 20 nm (z-average equal to 54.7 ± 3.2 nm) and 40 nm (z-average equal to 64.2 ± 2.6 nm) PS-NPLs, the z-average hydrodynamic diameters of 60 nm (75.0 ± 3.1 nm) and 100 nm (100.8 ± 1.9 nm) PS-NPLs were found closer to their primary size. This indicates that due to the chemical challenge in the synthesis of small nanoplastic particles or/and the combined effect of electrostatic repulsion and van der Waals force (Hamaker constant) (Bundschuh et al., 2018), small-sized NPLs are found here more polydisperse or more prone to aggregation than the large-sized ones in ultrapure water.

3.2. Behavior of PS-NPLs in lake water and in mineral water

To understand the role of key factors on PS-NPLs toxicity, detailed evaluation of the stability of PS-NPLs in the different freshwater conditions was analyzed via ζ-potential (Fig. 2 A–D) and z-average hydrodynamic diameter variations (Fig. 3 A–D) from 0 to 48 h. In mineral water (pH 7.2 ± 0.1), ζ-potential of all tested PS-NPLs showed positive values, ranging from

![Fig. 2. ζ-Potential of PS-NPLs of 20 nm (A), 40 nm (B), 60 nm (C), 100 nm (D) at 20 mg L⁻¹ in mineral water (green line) and lake water (blue line) as a function of time.](image-url)
+28.8 ± 1.3 mV to +43.2 ± 1.8 mV. After PS-NPLs dispersion in mineral water, their ζ-potential quickly stabilized after a brief decrease. This can be explained as mineral water mainly contains salt ions with positive and negative charges, including Ca^{2+}, Mg^{2+}, K^+, Na^+, HCO_3^−, SO_4^{2−}, PO_4^{3−}, Cl^− (Table S1). Electrostatic interactions and screening effects rapidly occur between the positively charged PS-NPLs and negative charges salt ions. Since these negative charges are insufficient to neutralize the positive charges on the surface of PS-NPLs, the surface charge of PS-NPLs remains positive. This explains why the z-average hydrodynamic diameters of PS-NPLs in mineral water is more stable than in lake water.

In lake water (pH 8.1 ± 0.1), in addition to salt ions with positive and negative charges, it also contains negatively charged NOM (Table S2). During the 48 h test period, the ζ-potential decreases with the increase of the primary size of PS-NPLs (Fig. 2A-D). Although the ζ-potential of 20 and 40 nm PS-NPLs are found lower than those in mineral water, they still remain positive. The ζ-potential of 60 nm PS-NPLs is already close to 0, while the surface charge of 100 nm PS-NPLs is reversed to negative values. All tested PS-NPLs are found unstable in natural lake water and rapidly form hetero-aggregates with sizes over 1000 nm after 30 min (Fig. 3 A-D). This is due to the electrostatic interaction between positively charged PS-NPLs, negatively charged salt ions and NOM, rapidly leading to charge neutralization and formation of large-diameter hetero aggregates. Among them, 20 and 40 nm PS-NPLs have larger specific surface areas and higher surface charges, so that the hetero-aggregates can still have positive charges. For 60 and 100 nm PS-NPLs, the amount of negative charges introduced by salt ions and NOM approached or even exceeded the positive charges carried by PS-NPLs, resulting in rapid charge neutralization or even charge reversal.

SEM was used to provide key visual information on the distribution and morphology of PS-NPLs in two exposure media. As shown in Fig. 4, 20 nm and 100 nm PS-NPLs exhibited a good stability in ultrapure water (Fig. 4-A & D). Once in mineral water, 20 nm and 100 nm PS-NPLs (Fig. 4-B & E) are observed to form a few of small-sized hetero-aggregates and to exhibit stable chain-like arrangement. This could be attributed to the higher ionic strength of the mineral water (Reynaud et al., 2022) and to the “bridging effect” of divalent cations especially Ca^{2+} and Mg^{2+} ions (Gutierrez et al., 2010; Shams et al., 2020). Furthermore, stronger changes in the behavior of PS-NPLs are observed in lake water (Fig. 4-C & F), compared to mineral water. Due to remarkable concentration of salt ions for example Ca^{2+}(1.03 μM), Mg^{2+}(0.24 μM) and the significant presence of NOM (0.96 mg L^-1 TOC), large-sized aggregates of both 20 nm and 100 nm PS-NPLs could be formed via electrostatic interaction and steric interactions (Saleh et al., 2008; Shams et al., 2020; Wu et al., 2019). Overall these observations indicate that PS-NPLs could follow different aggregation mechanism in different exposure conditions.

Based on the above observations on the stability of the different 4 primary sizes PS-NPLs and chemical composition of freshwater conditions, hetero-aggregation mechanisms are schematically proposed in Fig. 5. In lake water, electrostatic interactions between PS-NPLs, negatively charged salt ions, and NOM rapidly lead to charge neutralization and the formation of large hetero aggregates. The z-average diameters of these complex aggregates formed by four PS-NPLs are as follows: 20 nm (9130 ± 3620 nm) > 60 nm (8517 ± 3900 nm) > 100 nm (6048 ± 1170) > 40 nm (3977 ± 1230 nm). Among them, 20 nm PS-NPLs induced the largest diameters, which exhibited 1.5 times than 100 nm PS-NPLs. Fig. 3. Z-average hydrodynamic diameter of PS-NPLs of 20 nm (A), 40 nm (B), 60 nm (C), 100 nm (D) at 20 mg L^-1 in mineral water (green line) and lake water (blue line) as a function of time.
Fig. 4. SEM image of 20 nm and 100 nm PS-NPLs in Ultrapure water (A & D), in mineral water (B & E) and in lake water (C & F). Scale bars: 100 nm.

Fig. 5. Hetero aggregation mechanisms occurring for the 4 sizes PS-NPLs in different media (ultrapure water, mineral water and lake water) after 24 h exposure.
These results are consistent with those reported by previous studies investigating the size-effect on the aggregation behavior of PS-NPLs, which indicate that the smaller PS-NPLs may have a higher aggregation rate compared with larger ones (Li et al., 2021a) via electrostatic and steric effects arising from NOM corona formation (Li et al., 2021b; Liu et al., 2013). Besides the primary size effect, the aggregate size induced by 60 nm PS-NPLs exhibited 2.1 times than that induced by 40 nm PS-NPLs due to their surface charge being closer to the isoelectric point. This finding agreed with a previous study which reported that the charge neutralization mechanism is responsible for the formation of large hetero aggregates (Oriekhova and Stoll, 2018), and highlighted the necessity of accounting for particle size and surface charge when evaluating the aggregation state of PS-NPLs in natural aquatic environment.

3.3. Toxicity of PS-NPLs on *D. magna* and *G. fossarum* in mineral water and in lake water

To determine whether the observed differences in PS-NPLs stability behavior result in changes in toxicity to *D. magna* and *G. fossarum*, organism mortality was determined in lake water and mineral water. As shown in Fig. 6, four size of PS-NPLs induce statistically significant increase of organism’s mortality with a dose-response relationship. Moreover, the toxicity of PS-NPLs size ECso value was defined to provide biological criteria for better evaluate the difference in exposure media. As regards the *D. magna* ECso values, in lake water, increasing the primary size of PS-NPLs resulted in a decreasing trend in *D. magna* mortality in the following order: 100 nm (12.36 ± 0.20 mg L$^{-1}$) > 60 nm (7.62 ± 0.15 mg L$^{-1}$) > 40 nm and 20 nm (2.45 ± 0.12 mg L$^{-1}$) (Table S3). ECso value of 20 nm PS-NPLs is similar with 40 nm PS-NPLs, and the dose-response curves of the two almost overlapped (Fig. 6A). The toxicity of 40 nm and 20 nm PS-NPLs was about 5 times that of 100 nm PS-NPLs in lake water.

In mineral water, a similar size-toxicity trend was observed (Fig. 6B). *D. magna* ECso caused by four PS-NPLs were as follows: 100 nm (6.05 ± 0.13 mg L$^{-1}$) > 20 nm (1.47 ± 0.06 mg L$^{-1}$) > 60 nm (1.17 ± 0.09 mg L$^{-1}$) > 40 nm (0.46 ± 0.11 mg L$^{-1}$) (Table S3). In mineral water, 40 nm PS-NPLs induced the highest toxicity, which exhibited 13.2 times than 100 nm PS-NPLs in ECso values. These results are consistent with those reported by previous studies in *D. magna*, which indicate that the smaller-sized NPLs may have more prominent toxic effects than larger-sized NPLs (Kelpsien et al., 2020; Pochelon et al., 2021). Beside the primary size effect, the ECso values of PS-NPLs in mineral water exhibit 1.3–6.5 times higher than those in the natural lake water. This toxicity discrepancy could be due to the formation of eco-corona in the presence of NOM in Geneva lake water, which reduced the NPLs aggregation and consequently decreased the toxicity by preventing their surface interaction (Junaid and Wang, 2021; Nasser and Lynch, 2016; Saavedra et al., 2019). Moreover, smaller size NPLs have greater surface-to-volume ratio and higher surface reactivity than larger size NPLs, making them more sensitive by environmental factors (Bundschuh et al., 2018; Pochelon et al., 2021). These findings evidenced that the exposure condition is important factor on the toxicity of PS-NPLs.

Similar to *D. magna* neonates, the toxicity of four PS-NPLs also exhibited the dose-response, size-dependent and exposure condition-dependent trend in the 24 h *G. fossarum* acute toxicity tests (Fig. 6 C & D). *G. fossarum* ECso values in lake water was in the following order:

![Fig. 6. Dose-response curves for the effect of 4 size PS-NPLs on the mortality (%) of *D. magna* in lake water (A) and in mineral water (B); and *G. fossarum* in lake water (C) and in mineral water (D).](image)
60 nm (16.35 ± 0.20 mg L\(^{-1}\)) > 20 nm (14.24 ± 0.14 mg L\(^{-1}\)) > 100 nm (13.86 ± 0.08 mg L\(^{-1}\)) > 40 nm (4.07 ± 0.19 mg L\(^{-1}\)) (Table S4). The toxicity of 40 nm PS-NPLs was about 3.4 times that of 100 nm PS-NPLs and about 4.0 times that of 60 nm PS-NPLs in lake water. The perturbation effect of fine particulate organic matter (FPOM) in lake water could significantly decrease locomotor activity of adult *G. fossarum* (Andreï et al., 2016; Mehennaoui et al., 2016). These observations allow us to hypothesize that the formation of hetero aggregates between PS-NPLs and NOM in lake water might have the similar adverse effect on *G. fossarum* neonates, and subsequently perturbate their locomotion system.

There were also statistically significant differences between the PS-NPLs toxicity in mineral water and in lake water. PS-NPLs was at least 1.3 times more toxic in mineral water than in the lake water. EC\(_{50}\) values for mortality on *G. fossarum* in mineral water caused by four PS-NPLs sizes were as follows: 60 nm (10.95 ± 0.20 mg L\(^{-1}\)) > 20 nm (5.13 ± 0.04 mg L\(^{-1}\)) > 40 nm (3.12 ± 0.14 mg L\(^{-1}\)) > 100 nm (1.35 ± 0.08 mg L\(^{-1}\)) (Table S4). 40 nm PS-NPLs still induced the highest toxicity, with 3.52 times higher than 60 nm PS-NPLs in EC\(_{50}\) value. 100 nm PS-NPLs induced <5% of *G. fossarum* mortality at the highest exposure concentration (20 mg L\(^{-1}\)). PS-NPLs was also studied on the other benthic invertebrates such as *Chironomus riparius*, *Heterocypris incongruens* (Heinlaan et al., 2020), and *Gammarus roeseli* (Götz et al., 2022), in the water with simple composition or moderately hard water, the results are in accordance with the low toxic effect of 100 nm PS-NPLs observed in this study. There are no studies on the adverse effects of 20 nm, 40 nm, and 60 nm PS-NPLs to freshwater benthic organisms in our knowledge. The significant adverse effects of 20, 40 and 60 nm PS-NPLs on the survival of *G. fossarum* in this study may be due to their smaller size causing them to be trapped in the guts, leading to food obstruction or leading to extra stress on other functions (Redondo-Hasselerharm et al., 2020; Redondo-Hasselerharm et al., 2021), or as the formation of large hetero aggregates lead to their adsorption on the *G. fossarum* carapace and pleopods, resulting in reduced locomotion activity or ventilatory activity (Mehennaoui et al., 2021), and finally leading to death.

Through light microscopy observation, after 24 h exposure of PS-NPLs in lake water, a large amount of black matter was observed in the intestine of *D. magna* (Fig. 7 - *D. magna*, Lake water. 20 nm–100 nm), but not in controls (Fig. 7 - *D. magna*, Lake water. Control). In mineral water, it was observed that not only a large amount of black matter accumulated in the intestine of *D. magna*, but also a high tendency to bind to organic surfaces, resulting in a large number of small flocs on *D. magna* as a “biological surface coating” as indicated by Dabrunz et al. (Dabrunz et al., 2011) (Fig. 7). This attachment phenomenon indicates that there are divalent cations (mainly Ca\(^{2+}\), Mg\(^{2+}\)) in mineral water can interact with the negative charges on the surface of *D. magna* to form small-sized aggregates. When PS-NPLs were added, they interacted with mineral ions to generate hetero aggregates, which could not only be absorbed by *D. magna* and accumulated in their intestine, but also adhered to their outer surface and caused them to molt prematurely (Fig. 7 - *D. magna*, Mineral water. 20 nm).

In *G. fossarum* test, the phenomenon of “biological surface coating” was also observed, especially in the presence of 20 and 40 nm PS-NPLs (Fig. 7 - *G. fossarum*, Lake water. 20 nm–100 nm).

![Fig. 7. Uptake and adsorption of 4 size PS-NPLs by *D. magna* and by *G. fossarum* in lake water and in mineral water after 24 h exposure. (Arrows indicate “biological surface coating”.)](image-url)
G. fossarum, Lake water and Mineral water. 40 nm). A large number of small flocs could be attached not only to G. fossarum carapace but also to their pleopods and gills, potentially leading to reduced G. fossarum locomotion (Haegerbaeumer et al., 2019; Zhao et al., 2017). This observed discrepancy between the toxicity from benthic and planktonic organisms highlighted the species specificity in the presence of PS-NPLs, and more research at cellular and molecular level will be needed to understand the mechanism of toxicity.

3.4. Correlation among intrinsic properties, exposure conditions and toxicity of PS-NPLs

To further examine the correlation among the intrinsic properties, exposure conditions, and toxicity of PS-NPLs, key parameters of complex colloidal dispersion system were selected to characterize the tested PS-NPLs: ζ-potential, z-average hydrodynamic diameter, conductivity, polydispersity index (PDI), pH, and corresponding EC50 value which constitute the variables of principal component analysis. According to Fig. 8 (A & C), the test PS-NPLs in lake water and in mineral water can be summarized as two different systems, exhibiting common characteristics. In addition, these parameters of PS-NPLs with different primary sizes were also classified and analyzed in lake water and mineral water, respectively (Figs. S1, S2, S3 and S4). It was found that PS-NPLs can be classified into four groups in the same exposure condition, which was consistent with their primary sizes. Although the behavior and toxicity of the same primary size PS-NPLs were strongly changed with different exposure times, they are characterized quite similarly in the PCA procedure. Overall, these findings reveal that the behavior and toxicity of PS-NPL are primarily exposure-condition-dependent and size-dependent.

Moreover, the correlation analysis between the surface properties and ecotoxicity of all tested PS-NPLs in the 2 freshwater conditions on both organisms (Fig. 8 B & D) showed that the 24-h EC50 on D. magna was strongly negatively correlated with ζ-potential (p < 0.001, r = −0.79), significantly positively correlated with polydispersity index (p < 0.05, r = 0.54), and negatively correlated with conductivity (p < 0.05, r = −0.51). Surprisingly, the G. fossarum correlation analysis showed different information. The 24-h EC50 on G. fossarum was strongly positively correlated with z-average hydrodynamic diameter (p < 0.05, r = 0.76), while it had no statistically significant correlation with ζ-potential. This result indicates that, once PS-NPLs enter the natural waters, they rapidly interact with
other particles and dissolved compounds to form a complex colloidal system composed of hetero aggregates. For planktonic and benthic organisms with different physiological traits, the ecotoxicity of NPLs may be determined by different factors in the system. For *D. magna*, the higher the ζ-potential and the more homogeneous dispersion of the hetero aggregates, the more significant acute toxic effects is on *D. magna*. For *G. fossarum*, the larger the ζ-average hydrodynamic diameter of the hetero-aggregates, the weaker acute toxic effects is on *G. fossarum*.

4. Conclusion
The aim of this study is to further derive informative links between the physicochemical properties of NPLs, environmental variables, and their resulting toxicological potential. For that purpose, PS-NPLs having different sizes in the nano range, two characteristic natural freshwater conditions, and two typical crustaceans were investigated. ζ-Potential, ζ-average hydrodynamic diameter, conductivity, polydispersity index (PDI), pH, EC50 were introduced as six key parameters to characterize these complex colloidal systems composed of PS-NPLs and given freshwater conditions. PS-NPLs with different initial sizes are found to induce a statistically significant mortality of *D. magna* and *G. fossarum* neonates with a dose-time response relationship, and exhibit 1.3–6.5 times higher toxicity in the mineral water than in the lake water. More importantly, ecotoxic effects are found driven by both the surface properties of PS-NPLs and environmental factors. The surface charge is also found the most influential toxicity factor of PS-NPLs on *D. magna* in environmental natural waters, while on *G. fossarum* the aggregation behavior is the stronger explanatory factor. The results leave open the question of the diversity of responses between species and the significance of natural water typologies on the aquatic invertebrates in the presence of particulate pollutants. The combination of the characterization of colloidal stability of nanoparticles and effect assessment in environmental natural waters systems in this work evidenced the relationship between colloidal stability of NPLs, exposure water properties, the physiological trait of organisms and the biological response. The future studies will focus on the exploration of mechanism understating of toxicity at cellular and molecular level. Our study demonstrates the necessity of considering in detail both NPLS intrinsic properties and media properties when assessing the biological effects of NPLs, and more species and types of natural water having contrasted chemical properties need to be considered for nanotoxicity assessment.

CRediT authorship contribution statement
Zijiao Meng: Methodology, Investigation, Characterization, Writing - original draft. Rémi Recoura-Massaud: Methodology, Investigation, Writing - review & editing. Arnaud Chaumont: Methodology, Investigation, Writing - review & editing. Serge Stoll: Conceptualization, Methodology, Validation, Supervision, Writing - review & editing. Wei Liu: Conceptualization, Methodology, Validation, Supervision, Writing - review & editing.

Data availability
Data will be made available on request.

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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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.158763.

References
Aili, O.S., Claveau-Mallet, D., Kururu, R.S., Lapointe, M., Bayen, S., Tufenkji, N., 2022. Weathering pathways and protocols for environmentally relevant microplastics and nanoplastics: what are we missing? J. Hazard. Mater. 423, 126955.
Andrej, J., Pain-Devín, S., Felten, V., Devin, S., Giambérini, L., Mehennau, K., et al., 2016. Silver nanoparticles impact the functional role of Gammarus roeseli (Crustacea Amphipoda). Environ. Pollut. 216, 608-618.
Boucher, J., Frilot, D., 2017. Primary Microplastics in the Oceans: A Global Evaluation of Sources.
Boyle, K., Örmeç, B., 2020. Microplastics and nanoplastics in the freshwater and terrestrial environment: a review. Water 12.
Bundschuh, M., Filser, J., Läderwald, S., Mcke, M.S., Metrevelli, G., Schönmann, G.E., et al., 2018. Nanoparticles in the environment: where do we come from, where do we go to? Environ. Sci. Eur. 30, 6.
Coyle, R., Hardiman, G., O’Driscoll, K., 2020. Microplastics in the marine environment: a review of their sources, distribution processes, uptake and exchange in ecosystems. Case Stud. Chem. Environ. Eng. 2, 100010.
Dahroun, A., Dauster, L., Prase, C., Seitz, F., Rosendfeldt, R., Schüle, C., et al., 2011. Biological surface coating and molting inhibition as mechanisms of TiO2 nanoparticle toxicity in Daphnia magna. PloS one 6, e20112.
El Hadri, H., Giganít, M., Matix, B., Grassl, B., Reynaud, S., 2020. Nanoplastic from mechanically degraded primary and secondary microplastics for environmental assessments. Nanoimpact 17, 100206.
Elkhatib, D., Oyane-del Gravez, V., 2020. A critical review of extraction and identification methods of microplastics in wastewater and drinking water. Environ. Sci. Technol. 54, 7037-7049.
Enfrin, M., Lee, J., Gibert, Y., Basheer, F., Kong, L., Dumé, L.F., 2020. Release of hazardous nanoplastic contaminants due to microplastics fragmentation under shear stress forces. J. Hazard. Mater. 384, 121193.
Esposito, G., Preso, M., Renzi, M., Anselmi, S., Cesaroni, A., Barceló, D., et al., 2022. Occurrence of microplastics in the gastrointestinal tract of benthic by-catches from an eastern Mediterranean deep-sea environment. Mar. Pollut. Bull. 174, 113231.
Gaylarde, C., Neto, J., Fonseca, E., 2020. Nanoplastics in aquatic systems - are they more hazardous than microplastics? Environ. Pollut. 272, 116950.
Geford, O., Xuereb, B., Chauvat, A., Geford, A., Biagiante, S., Noël, C., et al., 2010. Ovarian cycle and embryonic development in Gammarus fossarum: application for reproductive toxicity assessment. Environ. Toxicol. Chem. 29, 2249-2259.
Geyer, R., Jambeck, J.R., Law, K.L., 2017. Production, use, and fate of all plastics ever made. Sci. Adv. 3, e1700782.
González-Pleiter, M., Tamayo-Belda, M., Púlpido-Reyes, G., Amare, G., Legnés, F., Rosal, R., et al., 2019. Secondary nanoparticles released from a biodegradable microplastic severely impact freshwater ecosystems. Environ. Sci. Nano 6, 1382-1392.
Götz, A., Beggel, S., Geist, J., 2022. Dietary exposure to four sizes of spherical polystyrene, polylactide and silica nanoparticles does not affect mortality, behaviour, feeding and energy assimilation of Gammarus roeseli. Ecotoxicol. Environ. Saf. 238, 113581.
Giudoghi, S., 2018. Contamination of table salts from Turkey with microplastics. Food Addit. Contam. Part A 35, 1006-1014.
Gutiérrez, I., Mylon, S.E., Nash, B., Nguyen, T.H., 2010. Deposition and aggregation kinetics of rotavirus in divalent cation solutions. Environ. Sci. Technol. 44, 4552-4557.
Haegerbaeumer, A., Mueller, M.-T., Fueser, H., Traunspurger, W., 2019. Impacts of micro- and nano-sized plastic particles on benthic invertebrates: a literature review and gap analysis. Front. Environ. Sci. 7.
Hazaraz, L., Veinlay, G., Boxadina, M., Perna, S., Ceti, D., Suáreda, Z., et al., 2020. Investigation of the toxic effects of different polystyrene micro- and nanoparticles on microalgae Chlorella vulgaris by analysis of cell viability, pigment content, oxidative stress and ultrastructural changes. Mar. Pollut. Bull. 156, 112786.
Heinlan, M., Kaeunen, K., Arasjo, V., Blinova, I., Bondarenko, O., Lukjanova, A., et al., 2020. Hazard evaluation of polystyrene nanoplastics with nine biosayss did not show particle-specific acute toxicity. Sci. Total Environ. 707, 136073.
Ilízaga, M.E., Conesa, J.A., Fullana, A., 2017. Microplastics in Spanish table salt. Sci. Rep. 7, 8620.
Ivleva, N.P., 2021. Chemical analysis of microplastics and nanoplastics: challenges, advanced methods, and perspectives. Chem. Rev. 121, 11886-11936.
Juganoss, K., Ivash, A., Blinova, I., Mortimer, M., Kahru, A., 2015. Nano-Tox new and in-depth database concerning ecotoxicity of nanomaterials. Beilstein J. Nanotechnol. 6, 1788-1804.
Junaid, M., Wang, J., 2021. Interaction of nanoparticles with extracellular polymeric substancess (EPS) in the aquatic environment: a special reference to eco-corona formation and associated impacts. Water Res. 201, 117319.
Kähl, A., Eriksson, T., Trojsten, O., Eklund, T., Hansson, L.-A., Cederwall, T., 2020. Long-term exposure to nanoplastics reduces life-time in Daphnia magna. Sci. Rep. 10, 5979.
Khoshrauamad, M., Hassachi, P., Ashtiani, S., Walker, T.R., 2021. Toxic effects of polystyrene nanoplastics on microalgae Chlorella vulgaris changes in biomass, photosynthetic pigments and morphology. Chemosphere 280, 130725.
Kirstein, J.V., Hensel, F., Gomiero, A., Ioedachescu, L., Vianello, A., Wittgen, H.B., et al., 2021. Drinking plastics? – quantification and qualification of microplastics in drinking water distribution systems by μTIR and py-GC/MS. Water Res. 188, 116519.
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Koelmann, A.A., Mohamed Nor, N.H., Hermoen, E., Kooi, M., Mintenig, S.M., De France, J., 2019. Microplastics in freshwater and drinking water: critical review and assessment of data quality. Water Res. 155, 410–422.

Li, Y., Li, W., Jarvis, P., Zhou, W., Zhang, J., Chen, J., et al., 2020. Occurrence, removal and potential threats associated with microplastics in drinking water sources. J. Environ. Chem. Eng. 8, 104527.

Li, X., He, E., Jiang, K., Petjinenburg, W.J.G.M., Qiu, H., 2021a. The crucial role of a protein corona in determining the aggregation kinetics and colloidal stability of polystyrene nanoparticles. Water Res. 190, 116742.

Li, X., He, E., Xia, B., Liu, Y., Zhang, P., Can, X., et al., 2021b. Protein corona-induced aggregation of differently sized nanoparticles: impacts of protein type and concentration. Environ. Sci.: Nano 8, 1560–1570.

Liu, J., Legros, S., von der Kamm, F., Hofmann, T., 2013. Natural organic matter concentration and hydrochemistry influence aggregation kinetics of functionalized engineered nanoparticles. Environ. Sci. Technol. 47, 4113–4120.

MacNeil, C., Dick, J.T., Elwood, R.W., 1997. The trophic ecology of freshwater Gammarus spp. (Crustacea: Amphipoda): problems and perspectives concerning the functional feeding group concept. Biol. Rev. 72, 249–364.

Mao, Y., Ai, H., Chen, Y., Zhang, Z., Zeng, F., Kang, L., et al., 2018. Phytoplankton response to polystyrene microplastics: perspective from an entire growth period. Chemosphere 208, 59–68.

Mattsson, K., Jocic, S., Doverbratt, I., Hansson, L.-A., 2018. Chapter 13. In: Zeng, EYBT-MCiAE (Ed.), Nanoparticles in the Aquatic Environment. Elsevier, pp. 379–399.

Mehthouaoui, K., Georntzopoulou, A., Felten, V., Andreï, J., Garaud, M., Cambier, S., et al., 2016. Gammarus fossarum (Crustacea, Amphipoda) as a model organism to study the effects of silver nanoparticles. Sci. Total Environ. 566, 1649–1659.

Mehennaoui, K., Cambier, S., Mingué, L., Serchi, T., Guérol, F., Guteh, A.C., et al., 2021. Sub-chronic effects of AgNPs and AuNPs on Gammarus fossarum (Crustacea Amphipoda): from molecular to behavioural responses. Ecotoxicol. Environ. Saf. 210, 111775.

Microbiotests, 2019. Standard Operating Procedure: Crustacean Toxicity Screening Test for reproduction group concept. Biol. Rev. 72, 249–364.

Oriekhova, O., Stoll, S., Slaveykova, V.I., 2018. Heteroaggregation of nanoplastic particles in the presence of natural organic matter, clay, and heavy metals. Environ. Sci.: Nano 6, 2968–2976.

Saleh, N.B., Pfefferle, L.D., Elimelech, M., 2008. Aggregation kinetics of multiwall carbon nanotubes in aquatic systems and environmental implications. Environ. Sci. Technol. 42, 7963–7969.

Schür, C., Zipp, S., Thalau, T., Wagner, M., 2020. Microplastics but not natural particles induce multigenerational effects in Daphnia magna. Environ. Pollut. 260, 113904.

Shams, M., Alam, I., Chowdhury, I., 2020. Aggregation and stability of nanoscale plastics in aquatic environment. Water Res. 171, 115401.

Shen, M., Zhang, Y., Zhu, Y., Song, B., Zeng, G., Hu, D., et al., 2019. Recent advances in toxicological research of nanoplastics in the environment: a review. Environ. Pollut. 252, 511–521.

Singh, N., Tiwari, E., Khandelwal, N., Darbha, G.K., 2019. Understanding the stability of nanoplastics in aqueous environments: effect of ionic strength, temperature, dissolved organic matter, clay, and heavy metals. Environ. Sci.: Nano 6, 2968–2976.

Wu, J., Jiang, R., Lin, W., Ouyang, G., 2019. Effect of salinity and humic acid on the aggregation and toxicity of polystyrene nanoplastics with different functional groups and charges. Environ. Pollut. 246, 160–170.

Woodall, L.C., Sanchez-Vidal, A., Canals, M., Paterson, G.L.J., Coppock, R., Sleight, V., et al., 2014. Molecular evidence for further overlooked species within the Gammarus fossarum complex (Crustacea: Amphipoda). Hydrobiologia 721, 165–184.

Yan, Z., Xu, L., Zhang, W., Yang, G., Zhao, Z., Wang, Y., et al., 2021. Comparative toxic effects of microplastics and nanoplastics on Chlamydomonas reinhardtii: growth inhibition, oxidative stress, and cell morphology. J. Water Process Eng. 43, 102291.

Yan, F., Liao, J., Bao, L., Yang, Z., Wang, G., et al., 2021. Transgenerational toxicity of nanopolystyrene particles in the range of μg L−1 in the nematode Caenorhabditis elegans. Environ. Sci.: Nano 4, 2356–2366.