Humic acid alleviates the toxicity of polystyrene nanoplastic particles to Daphnia magna

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Humic acid alleviates the toxicity of polystyrene nanoplastic particles to *Daphnia magna*†

Olunyio Fadare, Bin Wan, *Liang-Hong Guo, Yan Xin,* Yu Yang*ab

With worldwide environmental accumulation of plastics and their recognized degradation into smaller particles, attention to their impacts on ecological systems and humans has been increasing recently. However, environmental factors and their impacts are seldom considered during their eco-toxicity evaluation. In this study, *D. magna* neonates were used to assess and compare the acute toxicities of polystyrene micro-plastic particles (MPPs) and nanoplastic particles (NPPs) in the absence and presence of humic acids (HAs), an important environmental factor in aquatic systems. Four stress response and detoxification genes (*CAT*, *GST*, *HSP70*, and *P-GP*) were used to characterize the toxic response of the neonates to the exposure. Our results showed that NPPs were much more toxic than MPPs in that 10 mg L⁻¹ NPPs induced over 70% of death in 96 h but MPPs (as high as 400 mg L⁻¹) caused no mortality under all tested conditions. More importantly, we revealed a potent protective role of HA against NPP toxicity at environmentally relevant concentrations. The effect was concentration dependent, as 50 mg L⁻¹ HA subdued the NPP (400 mg L⁻¹) toxicity effect completely. NPPs elicited the up-regulation of all examined genes while HA diminished the toxicity effect completely. NPPs elicited the up-regulation of all examined genes while HA diminished the change appreciably, further confirming its detoxifying role against NPP toxicity. Through fluorescence and dynamic light scattering measurements, we found that HA was adsorbed on NPPs and formed a corona, without causing agglomeration or precipitation, but changed NPP distribution in the *D. magna* neonates in a way similar to that of MPPs, leading to alleviated toxicity. Our results suggest that it is essential to consider environmental factors in evaluating and monitoring NPP toxicity as this presents a more relevant exposure state.

**Environmental significance**

Plastic debris accumulates in the environment and its disintegration into smaller particles raised great concerns recently on their potential impact on environmental health. Particles in nanoscale were known to be more toxic than bulky materials, but previous toxicity evaluation on nanoplastic particles (NPPs) seldom considered the influence of environmental factors, which in no small degree dictate the behavior and chemistry of these particles. This work reports one of the ubiquitous environmental factors (humic acid [HA]); by adsorbing on NPPs and possibly forming a HA corona, HA altered the interactions between NPPs and *D. magna*, leading to a significant reduction effect of NPP toxicity in *Daphnia magna*. This study underscores the importance of putting environmental factors into consideration when evaluating NPP toxicity.

**Introduction**

Plastics have been produced and used extensively worldwide for over a half-century. Since a large number of these materials are resistant to biodegradation, they can persist in the environment for many years, resulting in a huge amount of mismanaged plastics in the environment. On the other hand, some of the plastic debris disintegrates over time into smaller particles of various sizes and shapes under natural processes such as UV radiation and mechanical abrasion.

In addition to the direct introduction of micro- and nano-sized plastics into personal care and pharmaceutical products, abiotic degradation of these solid wastes into micro- and nano-sized particles was demonstrated recently. Their
impacts on the environment have raised growing concerns, especially on the health of the aquatic system, since more than 80% of the plastic detritus accumulates in open water systems. These smaller particles become more mobile, bio-available and highly dynamic, presenting a more serious threat than the bulk materials to aquatic organisms. Vast records of the presence and isolation of microplastics as well as their side effects on aquatic organisms have been documented.

Recently, nanoplastics (NPPs) have gained more attention, since lines of evidence demonstrated the tendency of NPPs to be more toxic than microplastics to freshwater animals. These findings are not surprising because particles within nanoscale possess very unique properties such as large surface area to volume ratio, high surface curvature which renders them highly mobile, high adsorption capability and the ability to permeate membranes. However, information is still lacking on the environmentally relevant concentration of NPPs due to the present limitation in the analytical procedure of detection, identification, quantification and monitoring in the environment. Notwithstanding, the toxicity of NPPs to D. magna has been studied under different simulated media and conditions, and various side effects were reported including neonate malformations, reduction in body size, alterations in reproduction, uptake and accumulation of particles through the gut’s epithelial barrier, reduction in feeding rate, body burdens and immobilization, inability to feed on algae and embryonic defects. In addition, the adsorption of phenanthrene, nickel and even secreted protein from D. magna on NPPs was shown to cause higher toxicity to Daphnia.

However, few studies took environmental factors into consideration in the toxicity evaluation, which makes the various findings inadequate for evaluating the risk associated with NPPs in the ecosystem. Without doubt, nanoparticles (NPs) including NPPs in the environment tend to acquire macromolecules on their surfaces, forming the so-called eco-corona. It has been shown that the eco-corona plays an important role in dictating the behavior and toxicity of NPs in environmental organisms by altering their uptake and removal dynamics. These macromolecules accessible to NPs in the environment include natural organic matter (NOM) and secreted biomolecules that constitute an important class of environmental factors. Previous studies showed various impacts of NOM on different NPs such as silver nanoparticles (AgNPs), zinc oxide (ZnO), iron(m) oxide (Fe₂O₃) and so on. Specifically, in D. magna, the toxicity reduction of NPs including titanium dioxide (TiO₂), fullerences (nC₆₀), graphene oxide (GO) and carbon nanotubes (CNTs) was illustrated, resulting from nano-bio interactions with NOM. Hence, it is imperative to understand the interaction between NOM and NPPs. In addition, since the phenotypes of environmental organisms are subject to influences by both their genotype and many environmental effects, it is reasonable to consider environmental factors as a variable in the response of organisms to NPPs. By doing so, the results will be more relevant to risk assessment and useful in policy making. As a major component of NOM, humic substances (HS) exist in both the terrestrial and the aquatic environments such as lake sediments, peats, brown coals, and shales. As a mixture, HS possess phenolic and carboxylic groups contributing to surface charge as well as reactivity, and for individual humic acids, there is considerable similarity overall. In fresh waters, HS levels vary from 1 to 15 mg L⁻¹ and have been shown to be one of the most important environmental factors influencing the chemistry, disintegration, fate and bioavailability of environmental pollutants including nanoparticles.

The aim of this study is to test the hypothesis that HS may influence the toxicity of plastics. To achieve this, we used humic acid to represent HS and polystyrene beads as the model to evaluate the toxicity of microplastics and nanoparticles to D. magna, a widely used freshwater invertebrate model. We exposed D. magna neonates to NPPs in the absence and presence of HA and examined the effects of HA on NPP suspensions in aqueous medium as well as ingestion and toxicity to D. magna. Our results demonstrated the protective role of humic acid in the toxicity of NPPs to D. magna. We also tried to understand the underlying mechanism of this phenomenon by examining the interactions between NPPs and HA. The results demonstrated that HA was adsorbed onto NPPs and rendered the NPPs to behave in a way similar to that of microplastic particles in D. magna, which were found to be less toxic than NPPs. This study revealed that the effects of this important component of natural waters cannot be overlooked in the toxicological study of NPP.

Materials and methods

Plastic particles

Micro/nanoplastic particles of polystyrene PS-NH₂ (both labeled and non-labeled) were obtained from Sigma Aldrich (St. Louis, MO, USA) as 1% (w/v) aqueous suspensions. The mean particle size ranged from 0.10–0.12 μm (S.D. ≤ 20%) to 1.0–1.3 μm (S.D. = ±3–8%) in diameter according to the manufacturer’s technical information. The densities were 1.055 g cm⁻³ (micro-particles) and 1.03–1.07 g cm⁻³ (nano-particles). Labeled plastic particles have a fluorescence excitation wavelength at 481 nm and emission at 644 nm. All plastic particles were characterized using Fourier-transform infrared (FT-IR) spectrometry (FT/IR-6100, JASCO Corporation, Tokyo, Japan) to identify the surface functional groups and scanning electron microscopy (SU 8020, HITACHI, Tokyo, Japan) to examine the topography (surface/texture) and morphology. Dynamic light scattering was carried out to measure the hydrodynamic diameters and zeta potentials using a Zetasizer (Nano-ZS, Malvern Instruments, Worcestershire, UK).

Humic acid preparation

Suwannee River humic acid III (SRHA) standard was purchased from the International Humic Substances Society...
Preparation of administered plastic particles

The purchased particle suspensions were subjected to ultracentrifugation (Optima L-100K, Beckman Coulter, Indianapolis, IL, USA) for 1 h at 35,000 rpm at 4 °C to precipitate the plastic particles. The resulting pellet was re-suspended in Milli-Q water and the process was repeated three times to remove the surfactant and preservatives. The particles were finally re-suspended in the culture medium and placed in an ultrasonic bath for 30 min before use. The stock solutions of the particles were all adjusted to 1 g L⁻¹ in the culture medium and stored at room temperature. For exposure, the stock solutions were diluted to the desired concentrations with the culture medium.

Culture of Daphnia magna

D. magna strain was obtained from the University of Birmingham, United Kingdom (Bham 2) with a history of over 10 years of culture in the laboratory. D. magna were cultured in glass beakers in an aerated incubator according to OECD guidelines. In detail, the animals were fed with green algae (Chara vulgaris) once daily (2.5 × 10⁶ cells per L) at a constant temperature of 20 °C with a light–dark cycle of 14:10 h. The culture medium was replaced twice a week. Chara vulgaris were cultured in a standard BBM solution (Phytotechnology Laboratories, Shawnee Mission, KS, USA) at 28 °C under constant aeration and 24 h illumination. The cultured algae were pelleted by centrifugation at 4500 rpm at 4 °C for 5 min and were then re-suspended in Milli-Q water. The optical density (OD) of the algae was measured at 440 nm to give an absorbance of approximately 0.8 using a UV-Vis spectrophotometer (Agilent 89090A, Santa Rosa, CA, USA) before feeding. All experiments were carried out using <24 h neonates from the third generation based on the OECD recommendation. Enough neonates were collected from multiple adults and randomly separated into different beakers for exposure tests.

D. magna exposure and 96 h acute toxicity assay

Fifteen neonates were placed in each beaker and three beakers were set as a group for the treatment. Unlabelled MPP and NPP solutions of varied concentrations from 1 to 400 mg L⁻¹ were used for the exposure. OECD guidelines for the acute toxicity test of Daphnia were adopted. No food was administered throughout the experimental period (0–96 h). Immobilization or mortality was monitored at 24 h intervals. In parallel, to investigate the influence of HA on the toxicity of plastic particles, the toxic response of D. magna to NPPs was also recorded in the presence of humic acid with the following experimental setup: 5 mg L⁻¹ of HA was introduced into different concentrations of NPPs (1, 10, 50, 100, 200 and 400 mg L⁻¹). Varying concentrations (1, 5, 10, 20 and 50 mg L⁻¹) of HA were introduced into 400 mg L⁻¹ NPP suspensions for exposure tests.

Total RNA isolation and real-time reverse transcription PCR

For exposure assays, 1200 neonates from a pool of harvested offspring were randomly divided into 24 groups and exposed to 200 mg L⁻¹ NPP, 5 mg L⁻¹ HA, and a mixture of 200 mg L⁻¹ NPPs and 5 mg L⁻¹ HA (NPP + HA). Neonates in the culture medium were set as the control group. All treatments were performed in six replicates under the same experimental conditions as described above. For total RNA isolation, neonates were collected and submerged in Trizol reagent (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) before homogenization using an auto-sampling rapid grinding machine (Retsch MM 301, Haan, North Rhine-Westphalia, Germany) at 30 m s⁻¹ for 3 min and the resultant homogenates were transferred into new centrifuge tubes. The guanidinium thiocyanate–phenol–chloroform extraction method was employed according to the manufacturer’s instruction. Total RNA isolated was quantified and the purity assessed on a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). Thereafter, reverse transcription was carried out using a RevertAid First-strand cDNA synthesis kit (Thermo Fisher Scientific, Vilnius, Lithuania) with oligo(dT) as the primer. Quantitative PCR was performed using a qPCR kit (Promega, Madison, WI, USA) according to the manufacturer’s instruction with the following thermocycle: initial denaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, primer annealing and elongation at 60 °C for 30 s with 40 cycles. The primer sequences used for qPCR are shown in Table 1. β-Actin was used as the endogenous control, and gene expression fold changes over the control group were calculated using the 2⁻ΔΔCt method. All reactions were carried out in six replicates.

Fluorescence spectroscopy

Excitation/emission matrices (EEMs) of NPPs (400 mg L⁻¹), HA (50 mg L⁻¹) and NPP + HA were collected by scanning the samples at the excitation/emission wavelengths of 340–380/420–490 nm using a fluorometer (Fluoromax-4, Horiba Co., Edison, NJ, USA). The bandwidths for excitation and emission were both 3 nm, emission wavelength increment was 1 nm, and integration time was 0.5 s. In addition, the mixtures composed of various concentrations of HA (1–50 mg L⁻¹) and NPPs (400 mg L⁻¹) were centrifuged at 35,000 rpm for 1 h at 4 °C to precipitate the NPPs, after which the fluorescence intensity of both the supernatants and the NPP pellets (resuspended in Milli-Q water) was measured at the wavelengths of 360/490 nm for excitation/emission.
Statistical analysis was carried out using IBM SPSS Statistics 20.0 software. One-way analysis of variance (ANOVA) with LSD post hoc tests was used to evaluate the significant differences between the exposure group and the control. Experimental data were reported as means ± standard deviation (S.D.).

<table>
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<tr>
<th>Gene</th>
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<th>Reverse primer (5′-3′)</th>
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<td>TTCGCCAGATGTATGCTCC</td>
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### Results and discussion

#### Particle characterization

The particle suspensions appeared clear and colorless at varying concentrations between 10 mg L<sup>−1</sup> and 400 mg L<sup>−1</sup>, and no precipitates were observed throughout the experimental period (Fig. 1A and B). SEM imaging showed a shape of uniform spheres for both MPPs and NPPs (Fig. 1C and D). We estimated the average diameter of MPPs and NPPs to be ~1 μm and 0.1 μm (insets), respectively. (E) and (F) Typical FT-IR spectra of MPPs and NPPs showing their characteristic peaks of polystyrene.

![Photographs of MPP (A) and NPP (B) suspensions of various concentrations (10–400 mg L<sup>−1</sup>) in the constituted media after allowing to stand for 96 h, without any observed precipitation. SEM images of MPPs (C) and NPPs (D) showed uniform spheres with an average diameter of ~1 μm and 0.1 μm (insets), respectively. (E) and (F) Typical FT-IR spectra of MPPs and NPPs showing their characteristic peaks of polystyrene.](image-url)
μm and ~100 nm, respectively (Fig. 1C and D, inset) using Nano Measurer 1.2.5 software for image analysis and plotted using OriginPro 8 with twenty different images each and over four hundred particles for each sample. FT-IR analyses showed standard spectra of polystyrene (Fig. 1E and F) with characteristic peaks for both MPPs and NPPs at 651–787 cm\(^{-1}\) and 656–784 cm\(^{-1}\) for the aromatic C–H deformation vibration, 1421–1508 cm\(^{-1}\) and 1420–1500 cm\(^{-1}\) for the aromatic C–C bond stretching, 2817–3000 cm\(^{-1}\) and 2824–3001 cm\(^{-1}\) for the aromatic C–H asymmetric and symmetric tension, respectively. The zeta potentials of the MPPs and NPPs suspended in the constituted medium at various concentrations were between −26 and −30.5 mV and −7 and −27 mV, respectively (Fig. S1A and C and S2A and C†). The hydrodynamic diameters appeared stable with the increase in concentration (Fig. S1B and D and S2B and D†). We therefore speculate that the possible influence of the culture medium used in this study was the primary factor responsible for this observation. We rationalize that at lower concentrations, more ions in the culture medium neutralize the surface charge of NPPs (higher ion/NPP ratio), giving room to particle aggregation, whereas as the concentration of the NPPs increases with fixed concentration of culture medium (lower ion/NPP ratio) this effect is reduced due to the limited availability of these ions resulting from the increase in the NPP concentration in suspension. A similar trend was previously observed by Wu et al.\(^{43}\) Labeled MPPs and NPPs displayed similar morphological features and spectra to those of non-labeled ones (Fig. S2†) except for the presence of the fluorescence label.

**Acute toxicity test of MPPs and NPPs**

We evaluated and compared the acute toxicity of MPPs and NPPs to *D. magna* neonates using a 0–96 h acute toxicity assay. From the results of the assay (Fig. 2A), it was evident that MPPs showed no toxicity to the animals even at the highest concentration of 400 mg L\(^{-1}\) throughout 96 h of exposure, which is in accordance with the observation of previous studies using the same model.\(^{12}\) However, a significant toxicity (65% mortality) of NPPs was observed after 96 h of exposure at 10 mg L\(^{-1}\) concentration and (100% mortality was

![Figure 2](image-url)
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recorded) after 96 h of exposure at 200 mg L\(^{-1}\) and 400 mg L\(^{-1}\) There is a clear dose- and time-dependent response in D. magna exposed to NPPs (Fig. 2B).

With this result, we decided to focus our attention on the effect of HA on the toxicity of NPPs by introducing 5 mg L\(^{-1}\) HA to NPPs of varying concentrations in a similar setup. The concentrations of HA were demonstrated to be safe to D. magna neonates even by a 21 day chronic toxicity test, in which neonates were exposed to 5 mg L\(^{-1}\) and 50 mg L\(^{-1}\) HA concentrations and their molting rate, mortality and fecundity were cautiously monitored. No significant change was found in all the parameters during the assay (Fig. S3†). To our surprise, the toxicity of NPPs was dramatically decreased by HA. The neonates experienced 100% survival up to 72 h at concentrations between 10 and 200 mg L\(^{-1}\) NPPs, while only 30% mortality was recorded at the highest concentration (400 mg L\(^{-1}\)) of exposure after 96 h. This is in contrast to the 100% mortality recorded after 72 and 96 h in the group treated with NPP suspension in the absence of HA (Fig. 2C vs. B), indicating a strong influence of HA on NPP toxicity to the freshwater animals under study. To further confirm this protective role of HA against NPP toxicity, we varied the concentrations of HA (1, 5, 10, 20 and 50 mg L\(^{-1}\)) with the highest concentration of NPPs (400 mg L\(^{-1}\)), considering the environmentally relevant concentrations of HA. The results showed that with increasing HA concentration, there is a corresponding increase in the percentage survival of the animals. During 96 h of exposure, as low as 1 mg L\(^{-1}\) HA increased the survival rate by 12%, and 50 mg L\(^{-1}\) HA inhibited the toxicity of NPPs completely. It is worth noting that 10 mg L\(^{-1}\) HA could provide over 90% protection against NPP toxicity throughout the experiment (Fig. 2D), and this level of HA is commonly found in open waters, suggesting that NPPs in realistic scenarios may be much less toxic than reported previously.\(^{15,18,22,23,44–49}\) Likewise, Wu et al. recorded the survival rate of D. magna exposed to 20 mg L\(^{-1}\) of three different types of PS with different surface functions ranging between 15 and 95 ± 10% in the presence of HA.\(^{43}\) Similar reduction effects of humic substances on some NPs’ toxicity to D. magna were reported and different reasons have been attributed to this detoxification process. This includes regulation of the zeta potential and particle size,\(^{34}\) altered particle surface, inhibition of ion or nanoparticle dissociation,\(^{26,27,32}\) increased electrostatic repulsion forces and high aromaticity/phenolic content\(^{31}\) as well as reduction in generation of reactive oxygen species (ROS).\(^{29,30}\) It is imperative to note that of all these observations, surface modification and electrostatic repulsion by HA are likely phenomena in NPPs.

Our finding is critical to understanding the fate, bioavailability and possible threat NPPs pose to the freshwater system since HA forms a substantial constituent of the aquatic environment. HA was shown at cellular level to influence the passage of ions\(^{37}\) and the permeability of biological membranes and passage of ions\(^{50}\) in D. magna and at animal level, and increase the ingestion of nanoparticles (NPs) such as AuNPs in solution which results in higher mortality in D. magna.\(^{44}\) On the contrary, our findings strongly suggest that HA played a protective role against NPP toxicity. Taken together, it can be easily deduced that it is essential to take into account environmental factors such as HA in the ecotoxicity assessment of NPPs as they might alter the properties and toxic profiles of NPPs.

Expression of genes related to stress response and detoxification in D. magna

In order to gain more insight into the toxicity of NPPs as well as the protective role of HA in D. magna, we measured the expression of a few genes in the animals after their exposure to 200 mg L\(^{-1}\) NPPs, 5 mg L\(^{-1}\) HA and a mixture of the two (NPP + HA). Four genes related to stress response and detoxification mechanisms were chosen to characterize the toxic response. Glutathione-S-transferase (GST), a phase II enzyme providing important protective mechanisms against ROS,\(^{51,52}\) was used as a biomarker for oxidative stress evaluation. As shown in Fig. 3A, NPPs alone elicited a substantial increase (11-fold) of GST expression in D. magna over the control group, suggesting that oxidative stress was induced by NPPs in the neonates. However, HA and NPP + HA treatment groups experienced no significant change of this gene expression, indicating that no oxidative stress occurred. Similarly and to a lesser degree, the expression of catalase (CAT), another gene whose major role is to protect cells against oxidative damage,\(^{53}\) was induced to 2.2-fold by NPPs alone, whereas no statistically significant (\(p > 0.05\)) induction was observed in HA and NPP + HA groups (Fig. 3B). Heat shock protein 70 (HSP70) belongs to a family of highly conserved proteins, which function as chaperones that normalize and re-fold denatured proteins, thereby averting the deleterious effects. The unique behavior of HSP70 activation with a change in temperature/stress has been utilized in environmental monitoring.\(^{54}\) Our results showed a dramatic increase (9.6-fold) of HSP70 gene expression upon NPP exposure (Fig. 3C), while those of the other two treatments are not significant. Since all three genes are recognized as antioxidant genes that are expressed in defense against various environmental stressors and unfavorable conditions in the aquatic system,\(^{55}\) a change of their expression would be an indicator of stress in D. magna. Our results showed a very consistent tendency that NPPs induced the expression of these genes, but the presence of HA efficiently diminished the changes, suggesting a protective role that HA played against NPP toxicity, which is in accordance with the results of our 96 h acute toxicity (Fig. 2C and D).

P-GP is an important member of detoxification mechanisms in hosts in that it prevents the cells from absorbing detrimental substances.\(^{56}\) The P-GP gene expression pattern, on the contrary, showed an up-regulated (23.1-fold) expression level in D. magna treated with HA, much higher than that observed in the NPP-treated group (13.7-fold) (Fig. 3D), while no significant expression change was observed in the NPP + HA group. Some studies have implicated HA in the induction of oxidative stress and severe fitness impairment in D. magna.\(^{57,58}\) Since P-GP in animals functions to transport...
an extremely wide variety of compounds, especially organic compounds, out of the body, it is not uncommon that the expression of P-GP is induced by HA as a protective physiological response to HA. However, to our surprise, it seems the interaction between HA and NPPs was actually responsible for the silence of this gene in the NPP + HA group, suggesting that *D. magna* was not under stress in the mixture, further indicating the important role of HA in modulating the toxicity of NPPs. Bearing that in mind, it would be of great interest and environmental significance to examine the joint effects of NPPs with various types of HA since there are great variations in HA composition and distinct HA fingerprints in natural freshwater environments from one location to another. More work on this aspect is guaranteed in the future.

**Interactions between NPPs and HA**

Since HA has a strong adsorption capability to nanoparticles in different media and conditions, there may be interactions between NPPs and HA, forming an eco-corona that might change the toxic behavior of NPPs. It has been shown that hydrophobic and π–π interactions are the major driving forces for the sorption of organic compounds to carbon-based nanoparticles. In addition, there is also a probability that HA interacts with NPPs through its carboxylic groups. The strong intermolecular attraction within the adsorbent layers may alter the surface property of NPPs in the suspension, and thus the toxicological profile. Fluorescence spectroscopy can establish the direct occurrence of interaction between microplastics and HA. We therefore examined the adsorption of HA on NPPs by obtaining the excitation/emission matrix (EEM) of HA solution, NPP suspension and NPP pellet precipitated out of the HA solution by ultracentrifugation (designated as pNPP/HA). As shown in Fig. 4A and B, NPP suspension and HA solution showed distinct EEM patterns, but pNPP/HA appeared to have a characteristic pattern similar to that of HA solution (Fig. 4C), indicating the presence of HA molecules on pNPP/HA. Note that the intensity scales differ and that the value of the blue to...
The aqua area of the EEM landscape of pNPP/HA is approximately equal to that of the brick red portion of the NPPs’ EEM, indicating the presence of NPPs. In the same vein, because HA appears brown in color and NPPs are pale white under visible light, we showed with the images (Fig. 4D) that after ultra-centrifugation, no apparent precipitation was observed for HA solution and a pale white pellet was obtained from the NPP suspension, whereas pNPP/HA appears to be brown, suggesting that a portion of HA molecules from the solution co-precipitated with pNPP/HA. Furthermore, by measuring the EEM of the supernatants, we found that after ultra-centrifugation, the supernatant of the NPP+HA mixture has obviously lower fluorescence intensities than that of the HA solution (Fig. 4E). We also observed a concentration-dependent increase of fluorescence intensity of pNPP/HA from the mixture of NPPs (400 mg L$^{-1}$) and various concentrations of HA (Fig. 4F). Taken together, all these data strongly suggest the adsorption of HA molecules on NPPs, forming the eco-corona.

Aggregation and precipitation play a significant role in the bioavailability and toxicity of particles in the aquatic system as these may influence their stability in solution.\textsuperscript{60} Aggregation increases the particle size, thereby reducing the total surface area,\textsuperscript{41} while precipitation of NPPs would possibly decrease the exposure risk of \textit{D. magna} to NPPs. Therefore, after confirming the interaction between HA and NPPs, we further measured and compared the zeta potentials and hydrodynamic diameters of NPPs in the presence and absence of HA. We found a slight decrease of the zeta potentials of NPPs in the presence of HA after 96 h (Fig. S1C vs. E$^\dagger$), but no increase in the hydrodynamic diameters was observed (Fig. S1D vs. F$^\dagger$). Different concentrations of HA did not significantly change the zeta potentials and the hydrodynamic diameters of NPPs (400 mg L$^{-1}$) (Fig. S1G and H$^\dagger$), suggesting that no agglomeration or precipitation of the NPPs occurred in the presence of HA, which is supported by previous studies.\textsuperscript{62–67} Hence, the sharp increase in the survival rate of \textit{D. magna} in the presence of HA could not have been a result of NPP aggregation or precipitation in solution.

\textit{D. magna} is a freshwater invertebrate feeding by effective water filtration. One can easily envisage that this may lead to either ingestion or adherence interaction between the animals and plastic particles or the combined effects. Interestingly, by using labeled MPPs and NPPs, we were able to track the plastic particles \textit{in situ} under a laser confocal fluorescence microscope and found a clear difference in the distribution pattern of MPPs and NPPs in \textit{D. magna}. MPPs were observed in the intestinal tracts of \textit{D. magna} (Fig. 5D and H), without traces of the particle outside the animal’s body or within its cavity. However, most of the NPPs were found trapped within the filter combs on the phyllopods in its carapace rather than being ingested by the animals (Fig. S4$^\dagger$ and 5B and F) when compared to the control (Fig. 5A) or 5 mg L$^{-1}$ HA alone (Fig. 5E). The patterns we observed were very consistent over various concentrations (100 and 400 mg L$^{-1}$) (Fig. 5). Remarkably, we found that in the presence of HA, the distribution pattern of NPPs was changed completely to resemble that of MPPs (Fig. 5C and G), indicating the tendency of NPPs to show behavior like that of MPPs, which was demonstrated to be non-toxic to \textit{D. magna} (Fig. 2). It has been reported that \textit{D. magna} have difficulty in ingesting particles below the size range of 0.24–0.64 \textmu m, resulting in NPPs trapped within the feeding apparatus of the animal instead of being ingested.\textsuperscript{12} This so-called body burden could result...
in immobilization in *D. magna* and may account for the toxicity of NPPs. Therefore, the change of NPP distribution pattern by HA in *D. magna* may provide an explanation for the detoxification mechanism of HA. It is interesting to note that the eco-corona formed by secreted proteins on NPPs increased the uptake and toxicity to *D. magna* whereas the HA corona showed an opposite effect, suggesting that eco-coronas formed by different biomolecules may pose distinct influences on the toxic profile of NPPs. Hence, it is essential to evaluate their influence in a case-by-case manner.

In conclusion, it was evident from our study that NPPs are more toxic in comparison with MPPs. Humic acid displayed a significant detoxifying property towards the toxicity of NPPs in *D. magna* through fluorescence spectroscopy and dynamic light scattering analyses. HA was found to interact with NPPs, forming a HA corona, without causing any observable aggregation or precipitation of NPP suspension. Instead, HA could change the distribution pattern of NPPs in *D. magna* to that of less toxic MPPs, which may be envisaged as a possible detoxification mechanism of HA. While further research into the environmental risk of NPPs is ongoing, we suggest the incorporation of a realistic natural exposure environment into the assessment and monitoring of the toxic effect of NPPs in freshwater medium as there are multiple heterogeneous reactions influencing the nature of plastic particles in natural waters, hence dictating their behavior. We recommend more and detailed studies on different environmental factors and coronas as well as pollutants co-existing with NPPs in the freshwater system as this will facilitate a realistic risk assessment of environmental NPPs.

**Conflicts of interest**

There are no conflicts of interest to declare.

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**References**

4. K. Duis and A. Coors, Microplastics in the aquatic and terrestrial environment: sources (with a specific focus on...
31 F. Seitz, R. R. Rosenfeldt, M. Müller, S. Lüderwald, R. Schulz and M. Bundschuh, Quantity and quality of natural organic matter influence the ecotoxicity of titanium dioxide nanoparticles, Nanotoxicology, 2016, 10(10), 1415–1421.
34 Y. Zhang, T. Meng, L. Shi, X. Guo, X. Si, R. Yang and X. Quan, The effects of humic acid on the toxicity of graphene
61 J. Gao, K. Powers, Y. Wang, H. Zhou, S. M. Roberts, B. M. Moudgil, B. Koopman and D. S. Barber, Influence of Suwannee River humic acid on particle properties and


