



Effect of salinity and humic acid on the aggregation and toxicity of polystyrene nanoplastics with different functional groups and charges[☆]



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ABSTRACT

Studies regarding the effect of environmental factors on the environmental behaviour and potential toxicity of nanoplastics (NPs) are limited but important. In this study, four polystyrene NPs with different functional groups and charges (PS, PS-COOH, n-PS-NH₂, p-PS-NH₂) were selected to investigate the effect of humic acid (HA) and salinity on their aggregation behaviour and toxicity. The results showed that salinity significantly accelerated the aggregation of the four NPs, while HA mainly exerted a stabilizing effect on the three negatively charged NPs. In contrast, the positively charged p-PS-NH₂ aggregated significantly at first but remained stable as HA concentration further increased. The joint effect mainly depended on their concentration ratio. The aggregation phenomena can be explained by the Derjaguin - Landau - Verwey - Overbeek (DLVO) theory. Also, the acute toxicity of NPs on *Daphnia magna* was affected by the surface charge of NPs, and the positively charged p-PS-NH₂ showed the lowest toxicity among the selected NPs. Furthermore, the presence of HA effectively alleviated the toxicity of PS and p-PS-NH₂, as the survival rates increased from 15% to 45%–95% and 100% respectively. Our results demonstrate that the surface properties of NPs significantly influence their aggregation and toxicity.

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1. Introduction

Various products containing plastic have many excellent properties, resulting in their rapid growth in production and inevitable disposal (Jambeck et al., 2015). Upon the release of plastic debris into the aquatic environment, fragmentation processes (Andrady, 2011; Barnes et al., 2009) such as weathering, biodegradation and photodegradation can generate microplastics (MPs, < 5 mm in diameter) (Arthur et al., 2009) and further nanosized plastics called nanoplastics (NPs) (Dawson et al., 2018). These small particles are considered a global threat due to their potential environmental toxicity (Besseling et al., 2014; Jahnke et al., 2017; Jemec et al., 2016). It has been reported that MPs and NPs were mistakenly ingested by aquatic organisms causing intestinal damage (Lusher et al., 2013) and cell damage (Wang et al., 2013) or acted as a

vector (Batel et al., 2016; Koelmans et al., 2016; Velzeboer et al., 2014) for other contaminants. The abundance of them has considered increasing exponentially (Cozar et al., 2014) compared to larger plastics debris. However, it has been reported that the sea surface lacks particles smaller than 1 mm (Cozar et al., 2014), suggesting ingestion by aquatic organisms (Cole et al., 2013), wind-induced vertical transport, (Reisser et al., 2015), or the formation of biofilms (Kooi et al., 2017; Rummel et al., 2017) etc., but the specific processes and mechanisms remain largely unknown.

As similar particulate contaminant, theories have been developed over a decade of studying engineered nanoparticles (ENPs) in the environment and may act as a cornerstone in studying the fate and exposure assessment of NPs (Hüffer et al., 2017). In regards to the ENPs study, dissolved organic matter (DOM) and ionic strength were important factors affecting the environmental behaviours of ENPs (Alimi et al., 2018; Hüffer et al., 2017). DOM can interact with ENPs through adsorption (Aiken et al., 2011; Chen et al., 2018, 2014), provide negative surface charges and change repulsive energy between particles according to the Derjaguin - Landau - Verwey - Overbeek (DLVO) theory (Bian et al., 2011; Zhang et al., 2009).

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Ionic strength (Ren et al., 2017; Zhang et al., 2009) can accelerate the aggregation process by compressing the electrostatic double layer (EDL) (Bian et al., 2011; Zhang et al., 2009). Previous studies also reported the effect of DOM and ionic strength on the aggregation of MPs/NPs (Cai et al., 2018; Li et al., 2018; Oriekhova and Stoll, 2018), however, no studies have focused on the effect of high salinity, which is a distinctive feature in estuaries, and the joint effect of salinity and DOM on the aggregation of NPs with different functional groups and charges.

An increasing number of studies have focused on the toxicity of NPs due to their small size-effect. It was said that smaller plastic debris is easier to be absorbed onto the organism due to their larger specific surface area (Brown et al., 2001). One study reported that smaller polystyrene (PS) microbeads were more toxic, as 6 μm microbeads were more effectively egested than 0.05 or 0.5 μm microbeads (Jeong et al., 2016). Also, the capability to adsorb and transport persistent organic pollutants allowed their ingestion and accumulation of contaminant in the aquatic ecosystem, thus increased the deleterious effects (Koelmans et al., 2016). Furthermore, the characteristics of NPs also affect their toxicity (Manfra et al., 2017; Nomura et al., 2013). Anionic PS-COOH formed microscale aggregates, showing no mortality, while cationic PS-NH₂ exerted the opposite effect on rotifer larvae (Manfra et al., 2017). Compared to negatively charged NPs, the positively charged PS-NH₂ exerted high cytotoxicity on yeast by neutralizing the surface charge on the cell membrane (Nomura et al., 2013). However, no research has investigated the variation in the toxicity of NPs when DOM is present.

This current work investigates the impact of different functional groups on the aggregation behaviour and toxicity influenced by high salinity and humic acid (HA). Four NPs with different functional groups, the polystyrene nanoplastics (PS), carboxyl-modified (PS-COOH) and oppositely charged amino-modified nanoplastics (n-PS-NH₂ and p-PS-NH₂) were selected and studied. The aggregation mechanism was interpreted using the DLVO theory. Furthermore, the relationship between aggregation and toxicity of NPs with and without HA was investigated using *Daphnia magna* which is a sensitive and important aquatic species in freshwater food chains.

2. Experimental section

2.1. Properties and preparation of materials

Four nanosized PS NPs (PS, PS-COOH, n-PS-NH₂ and p-PS-NH₂) were used to study aggregation behaviour. The PS, PS-COOH and n-PS-NH₂ suspensions, which were negatively charged, were obtained from Aladdin (Shanghai, China) with a concentration of 2.5% w/v. The p-PS-NH₂ (2% w/v), which was positively charged, was purchased from Thermo Fisher Scientific (Shanghai, China). The manufacturer of Aladdin (Shanghai, China) prepared PS, PS-COOH and n-PS-NH₂ suspensions in the solution with ethanol and de-ionized water (v/v, 1:1), while the manufacturer of Thermo Fisher Scientific (Shanghai, China) used de-ionized water as the medium for p-PS-NH₂ suspension, which is solvent-free and ultraclean according to the description on official website and the CERTIFICATE OF ANALYSIS. Their fundamental properties are presented in Table 1, and their FTIR spectra are provided in Fig. S1. The nominal size of PS in the vendor report is 100 nm, which is the average core size measured by a transmission electron microscope (TEM). However, the hydrodynamic diameter of the PS NPs measured by DLS is approximately 220 nm, which is intensity weighing for z-average hydrodynamic diameter. The TEM measurement only provides the core size, attributed to particle polydispersity (PDI) and average weighing (Baalousha and Lead, 2012; Regmi et al.,

2011). It is suggested that these two methods can obtain similar results only for highly monodisperse samples (i.e., PDI < 0.1). However, the PDI of PS and p-PS-NH₂ used are 0.280 and 0.203, respectively. Besides, fluorescent PS microspheres with a diameter of 100 nm (F-PS, 1% w/v) were also obtained from ThermoFisher Scientific (Shanghai, China). The maximum excitation and emission wavelength of F-PS is 365 nm and 447 nm presented in the vendor's report. The suspensions were stored at 4 °C in a refrigerator in the dark.

Humic acid (containing > 90% fulvic acid) purchased from Aladdin (Shanghai, China) was chosen as a representative of DOM. The total organic carbon (TOC) of the HA was measured by a Shimadzu TOC analyzer (Shanghai, China), and 0.419 mg L⁻¹ TOC was converted to 1 mg L⁻¹ HA. However, here all experimental results are expressed as HA concentrations. The elemental composition was determined using a Vario EL Elemental Analyzer (Elementar Corp., Germany) and is presented in Table S1. The HA stock suspension (1000 mg L⁻¹) was stored in the dark at 4 °C and diluted to 1–50 mg L⁻¹ for use in the experiments. A high salinity solution was prepared by dissolving solid NaCl (Guangzhou Chemical Reagent Co., Ltd., Guangzhou, China) into Milli-Q water.

2.2. Measurements of hydrodynamic diameters (D_h) and zeta potentials of NPs

The effect of HA and salinity, solely and jointly on the aggregation behaviour of PS NPs was first investigated by measuring their hydrodynamic diameter using an EliteSizer Omni instrument (Brookhaven Instruments Corp., New York, USA) at 25 °C by dynamic light scattering (DLS) with a 90° scattering angle. To study the sole effect of HA, a predetermined amount of HA stock solution was introduced to obtain concentrations of 0, 5, 10, 25, and 50 mg L⁻¹. To study the effect of salinity, the concentration of NaCl was adjusted to 0, 5‰, 10‰, 20‰, 30‰ and 35‰, to simulate real environmental conditions in an estuary. In the study of joint effects, the salinity concentration was fixed at 10‰ and 30‰ with HA concentrations ranging from 0 to 50 mg L⁻¹. We performed a control test to verify whether a high concentration of HA itself yielded signals in the DLS instrument. Results showed that HA itself would not create artefacts in our measurements of particle size and would not influence the conclusion obtained in this work. The NaCl solution was prepared first, and then an aliquot of the HA stock suspension was added. Subsequently, a small volume of concentrated NPs stock suspension was added to obtain the final NPs concentration of 300 mg L⁻¹. Finally, the mixture was sonicated for 15 min and placed on an orbital shaker for 24 h at a speed of 200 rpm before the test. The additives used to prepare the suspensions by the manufacturers would not influence aggregation tests in this work. The same amount of PS stock suspension was added in each experiment group. For exposure suspension with PS concentration of 300 mg L⁻¹, only 60 μL ethanol was introduced, which is negligible compared to the total volume of exposure suspension (10 mL). Also, since the amount of ethanol was constant for each treatment, the effect would be constant for the experimental groups. Zeta potentials of the NPs (100 mg L⁻¹) were measured using the same instrument by applying the laser Doppler electrophoresis method in combination with phase analysis light scattering (PALS) and multi-frequency measurement.

2.3. Fluorescence quenching of PS in the absence and presence of HA

The concentration of F-PS used in this test was consistent with the aggregation tests. The F-PS was added into the solutions containing HA concentrations of 5 and 50 mg L⁻¹, and then sonicated

Table 1
Fundamental properties of nanoplastics.

Nanoplastics	Shortened form	Size (nm)		Zeta potential (mV)
		Vendor report	DLS measurement	
Polystyrene nanoplastic	PS	100 nm	217.19±7.45	-27.13±0.47
Amino-modified polystyrene nanoplastic	n-PS-NH ₂	50–100 nm	92.79±0.96	-30.37±2.03
	p-PS-NH ₂	110 nm	192.88±2.30	48.91±2.13
Carboxyl-modified polystyrene nanoplastic	PS-COOH	300 nm	272.70 ± 4.20	-35.89±2.87

for 15 min and agitated on an orbital shaker for 24 h to make sure sufficient interaction occurred. A F97 Pro fluorospectro photometer (Shanghai Lengguang Technology Co., Ltd., Shanghai, China) was used to measure the fluorescence intensity of these samples. The excitation wavelength was 360 nm, and the maximum emission wavelength was 448 nm. We also took a photograph of the samples using a ZF-90D Dark Box Ultraviolet Analyzer (Shanghai Guanghao Analytical instrument Co., Ltd., Shanghai, China) under a 365 nm UV light source.

2.4. *Daphnia magna* acute toxicity test

A 48 h acute toxicity test on *Daphnia magna* was performed following the OECD guideline 202 (OECD, 2004). Tap water aerated overnight was used as the medium for the acute toxicity test because culture medium contains several types of ions that may impact the aggregation behaviour of NPs. Control groups were conducted in the aerated tap water without NPs and HA. The ethanol used to prepare the NPs suspensions was also added into the control group, and the amount (4 µL) was equal to the one introduced to the treated group.

The toxicity of the four NPs with a concentration of 20 or 30 mg L⁻¹ and HA with concentrations of 5, 10, 25, 50 mg L⁻¹ was tested both individually and jointly. Samples were prepared and incubated for 24 h, then sonicated for 15 min before *Daphnia magna* exposure. Five *Daphnia magna* (less than 24 h old) were exposed to different samples at 22 ± 0.5 °C with a 16 h:8 h (light:dark) photoperiod cycle. The immobilization and mortality data were recorded three times, at 0 h, 24 h and 48 h after exposure. The results are presented as 48 h survival rates.

2.5. Preliminary experiment

It is known that numerous factors such as exposure time, pH, humic acid concentration and salinity may influence the aggregation of NPs. We first examined which factors may exert a significant influence. Since a single-factor experimental design requires more experiments and it would be difficult to determine the main and interactive factors, the Box-Behnken Design (BBD) was used to identify the significant factors in the aggregation behaviour of PS. The detailed parameter settings and procedures are presented in the supporting information (SI).

The BBD experiment illustrated that HA concentration and salinity were two significant factors among the four selected factors. In addition, an interactive effect was observed between humic acid concentration and salinity (SI). In contrast, the other two factors, exposure time and pH, did not show a significant effect on the aggregation behaviour within the selected conditions. Therefore, the exposure time was kept to one day, and the pH ranged from 6.23 to 6.83 during the test. Furthermore, taking the environmental conditions into account, we narrowed the range of HA concentration to 0–50 mg L⁻¹ and the salinity to no more than 35‰.

3. Results and discussion

3.1. Effect of HA on the aggregation behaviour of NPs

Polystyrene is a synthetic aromatic hydrocarbon polymer made from monomer styrene and can be solid or foamed. It is one of the most widely used plastics, and the production is several million tons per year (Lithner et al., 2011). It was chosen as a type of representative plastics because it is one of the most abundant plastic types in marine debris (Plastics Europe, 2006) and account for 6–7.8% of the total plastic production worldwide (Lithner et al., 2011).

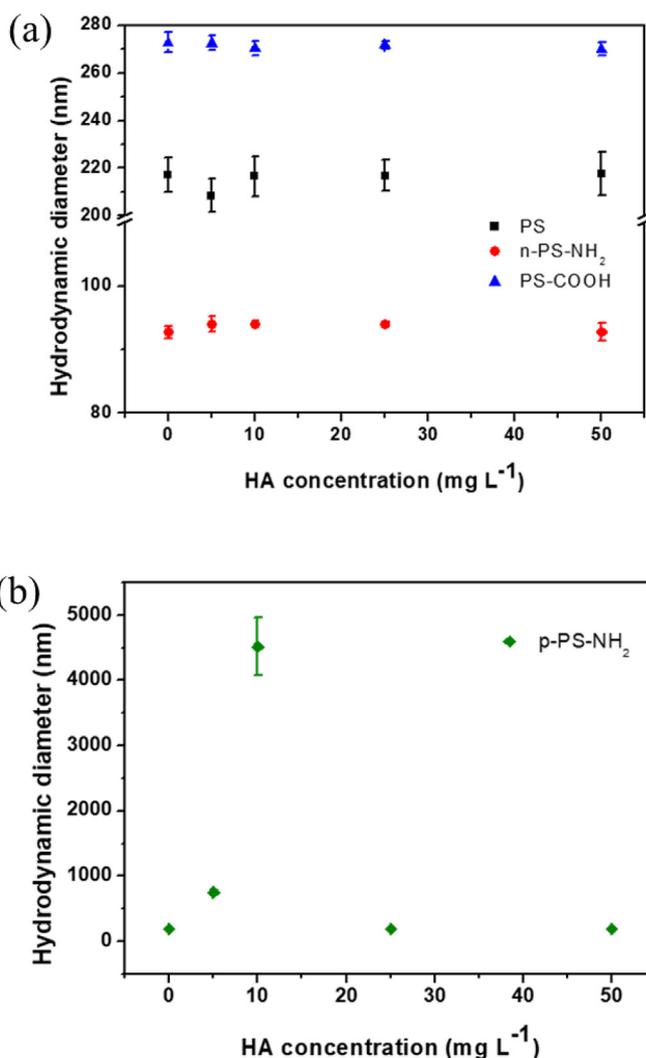


Fig. 1. Hydrodynamic diameters of PS, n-PS-NH₂, PS-COOH (a) and p-PS-NH₂ (b) in different HA concentrations. The error bars represent the standard deviation of 5 replicates.

In aqueous environments, the fate and transport of nanoparticles are connected to the adsorption of HA. The effect of HA on the aggregation of NPs was characterized by determining their hydrodynamic diameter. We used high NPs concentration (300 mg L^{-1}) to improve DLS data quality. Fig. 1 shows that HA alone had no significant effect ($P > 0.05$) on the aggregation of PS, n-PS-NH₂ or PS-COOH, while the hydrodynamic diameter of p-PS-NH₂ showed a sharp increase in the presence of low HA concentrations and decreased with further increases in HA concentration. To investigate the interaction between NPs and HA we studied the fluorescence change of a 100 nm fluorescent labelled microsphere suspension in the absence and presence of HA. The fluorescence intensity plot is presented in Fig. 2a, which demonstrates a decrease in fluorescence intensity as the added concentration of HA increased. The fluorescence intensity of the F-PS suspension without HA was 3758, and decreased to 3121 and 846.3 in the presence of 5 and 50 mg L⁻¹ HA, respectively. The photograph of the fluorescence is shown in Fig. 2b and indicates that HA adsorbed onto the F-PS surface and decreased the fluorescence intensity.

The negligible effect of HA towards the aggregation of PS, n-PS-NH₂ and PS-COOH may be a result of all of them having the same charge. After HA adsorbs onto the surface of the NPs, the adherence of HA provides more negative charges, causing it to remain in a stable state. On the other hand, when a positively charged particle meets negatively charged HA, the HA may first adsorb onto the particle and neutralize the surface charge, leading to an aggregation of particles. Experimental data also demonstrated this hypothesis. As shown in Fig. 4b, the 100 nm p-PS-NH₂ exposed

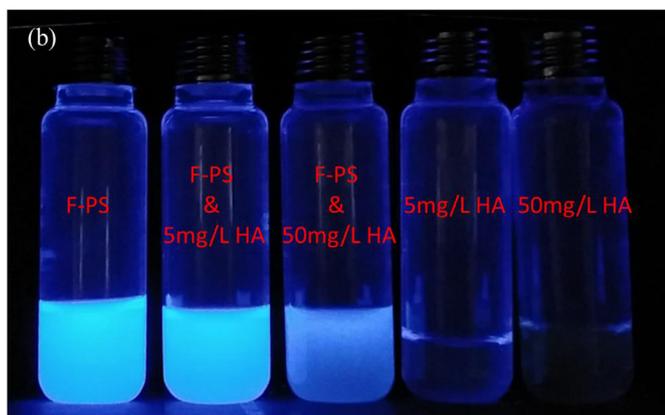
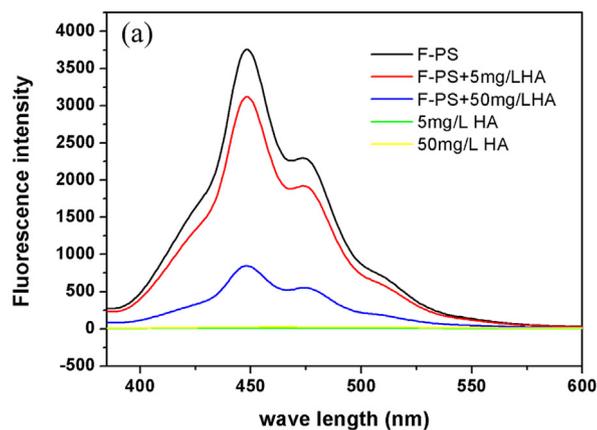


Fig. 2. (a) Quenching Fluorescence of F-PS in the absence and presence of 5 and 50 mg L⁻¹ HA. (b) Photograph of F-PS in the absence and presence of 5 and 50 mg L⁻¹ HA under 365 nm UV light source.

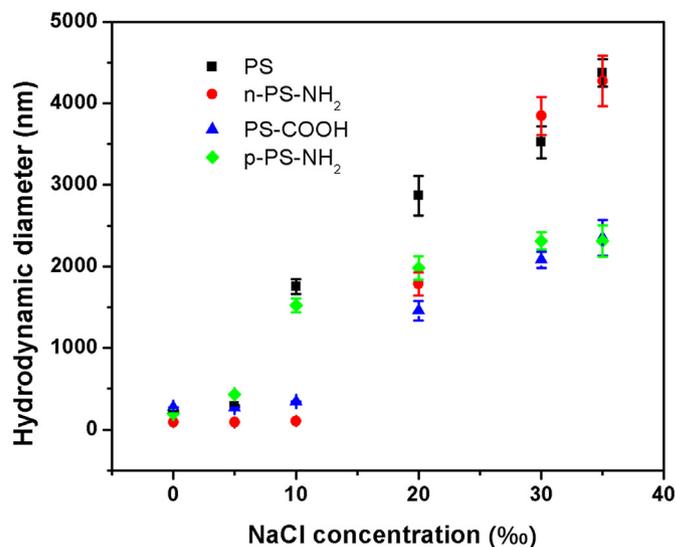


Fig. 3. Hydrodynamic diameters of four NPs in different salinity conditions. The error bars represent the standard deviation of 5 replicates.

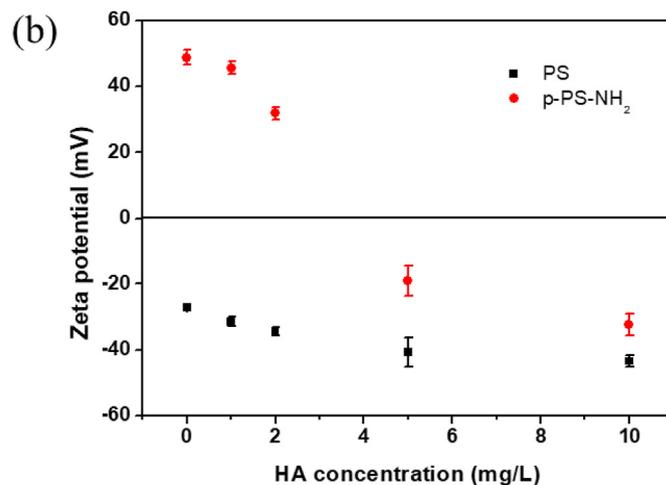
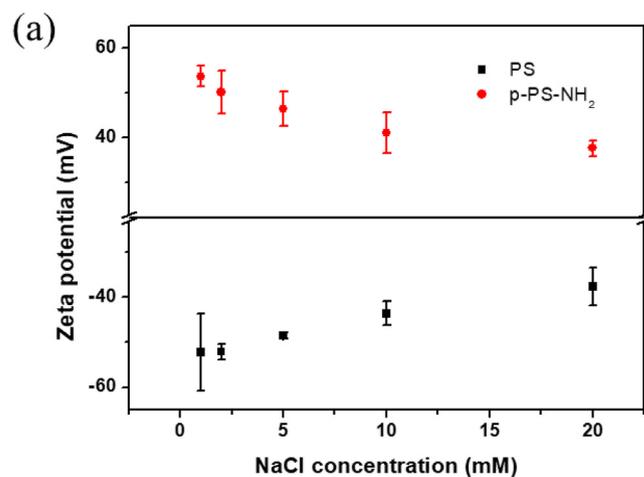


Fig. 4. Zeta potentials of PS and p-PS-NH₂ in different ionic strength conditions (a) and different HA concentrations (b). The error bars represent the standard deviation of 3 replicates.

to 10 mg L^{-1} HA showed strong aggregation and even reached a hydrodynamic diameter of 4516.01 nm. After further increasing the HA concentration, the extent of aggregation decreased to a hydrodynamic diameter of 192.04 nm and the stability of NPs was enhanced due to further adsorption of HA and consequently electrostatic repulsion and steric stabilization. This phenomenon is consistent with previous research by Jung et al. (2018) who investigated the aggregation of polyethylenimine (PEI) coated silver nanoparticles influenced by pony lake fulvic acid (PLFA). The aggregation is promoted by the adsorption of PLFA on the surface of positively charged nanoparticles but after further adsorption, the colloidal stability and dispersion enhanced due to electrostatic and steric hindrance.

3.2. Effect of salinity on the aggregation behaviour of NPs

The stability of nanoparticles in aqueous solutions was strongly affected by salinity. A salinity gradient from 0 to 35‰ was studied. As shown in Fig. 3, the hydrodynamic diameter of NPs in a solution with a low NaCl concentration (<5‰) did not show significant variation, but dramatically increased to 2000–4000 nm as the NaCl concentration became higher, which indicated a strong aggregation of four PS particles. In a recent report, Gigault et al. (2018) also found that PS nanoparticles were stable in the aqueous solution containing 350 mM of NaCl. When NaCl concentration was over 500 mM, PS started to aggregate irreversibly. The strong aggregation may lead to NPs accumulating in sediment and thus increase the potential risk to benthic organisms. Eduardo et al. (Acha et al., 2003) reported that “the distribution of debris was restricted to the freshwater and estuarine environments, showing highest concentrations in the riverine sector and in coincidence with the salinity front”, which act as a barrier accumulating debris. Cheung et al. (2016) also observed the influence of surface salinity on the transport and deposition of debris. Consequently, the salinity

gradient, which is a distinctive feature in estuaries, has a profound impact on the ecological consequences of NPs.

Zeta potentials of NPs with different salinities are shown in Fig. 4a. The zeta potential values of both negatively charged PS and positively charged p-PS-NH₂ decreased as the salinity increased. DLVO interaction energy profiles (Figs. S3a and b) showed that the interaction between NP particles at a low salinity was repulsive, resulting in a stable state. However, with an increase in NaCl concentration, the EDL can be compressed and thus decrease the repulsive forces, which results in a decreased net repulsive energy barrier between NP particles, causing aggregation. The detailed calculation formulas of the DLVO theory are presented in the SI.

3.3. The combined effect of HA and salinity on the aggregation behaviour of NPs

Many factors exist in the aqueous environment which has synergistic and antagonistic effects on the behaviour of nanoparticles (Akaighe et al., 2013; Besseling et al., 2017). Dissolved organic matter can adsorb onto the surface of nanoparticles, similar to a coating, and affect the stability even in a high ionic strength condition like a river or sea (Ghosh et al., 2010; Zhou and Keller, 2010). The effect of HA together with salinity on the aggregation behaviour of NPs as determined by hydrodynamic diameter is presented in Fig. 5. These DLS measurements show that the hydrodynamic diameter of PS and PS-COOH nanoparticles decreased as the concentration of HA increased in solution with 10‰ NaCl, while n-PS-NH₂ showed a slight increase. Both PS-COOH and n-PS-NH₂ were negatively charged but showed inverse aggregation trends, which may be due to the complex interaction between diverse functional groups on HA and NPs. In contrast, the hydrodynamic diameter of the positively charged p-PS-NH₂ reached a maximum value of approximately 4005.28 nm and a large aggregation formed when HA concentrations increased from 0 to

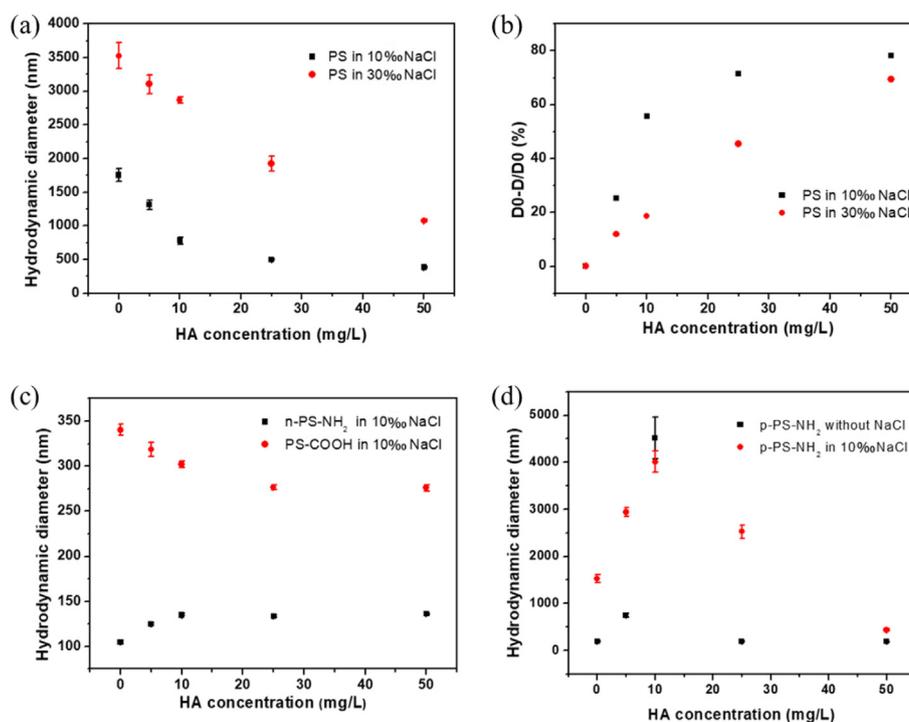


Fig. 5. Hydrodynamic diameters of PS (a), n-PS-NH₂, PS-COOH(c) and p-PS-NH₂ (d) in different HA concentrations and ionic strength conditions. (b) The relative reduction graph converted by Fig. 5a using D_0-D_h/D_0 to illustrate the reduction extent (D_0 : Hydrodynamic diameter of PS in the NaCl solution without HA; D_h : Hydrodynamic diameter of PS in different HA and salinity conditions.). The error bars represent the standard deviation of 5 replicates.

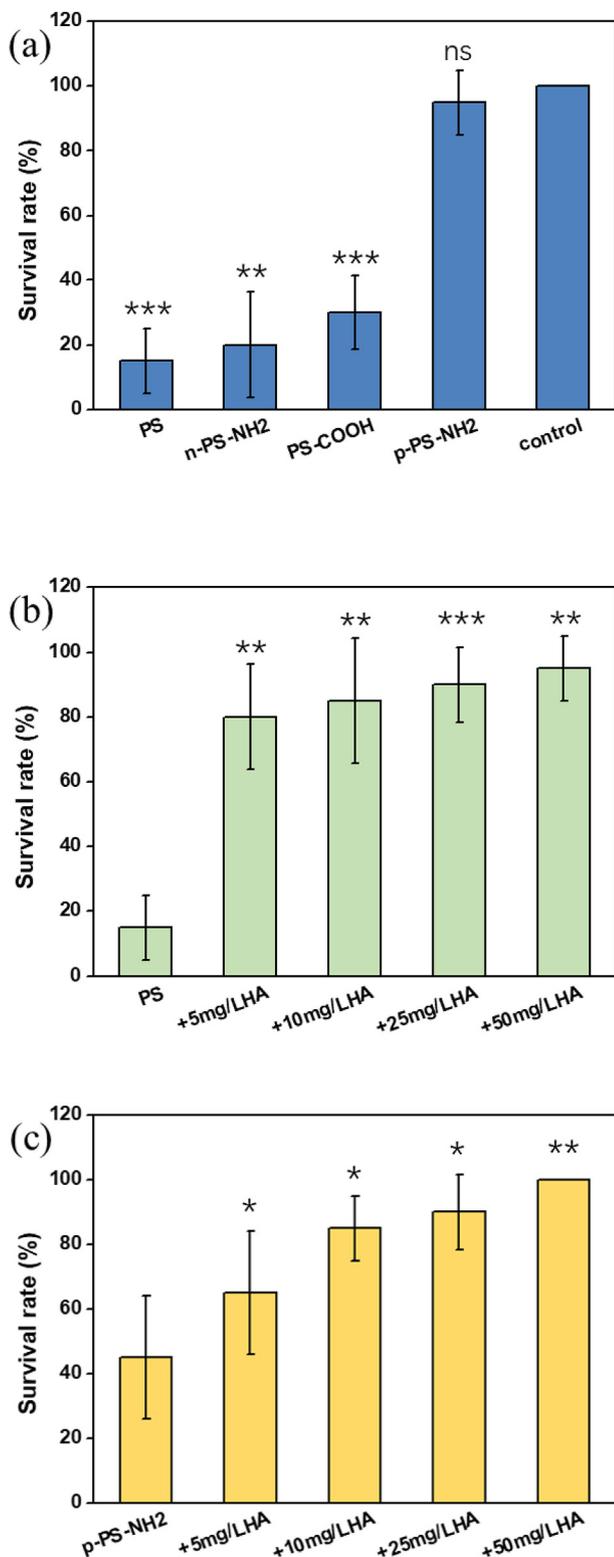


Fig. 6. 48 h survival rate (%) of *Daphnia magna* exposed to (a) the four NPs (20 mg L^{-1}) solely, (b) PS NPs (20 mg L^{-1}) and (c) p-PS-NH₂ (30 mg L^{-1}) in the presence of a series of HA concentrations. Mean values and standard deviations were calculated for 4 replicates. (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$ versus controls (Fig. 6a), PS (Fig. 6b) and p-PS-NH₂ (Fig. 6c).

10 mg L^{-1} . It then decreased to 431.47 nm when further HA concentrations were increased to 50 mg L^{-1} , and the suspension was stable.

According to zeta potential measurements presented in Fig. 4b, the zeta potential of p-PS-NH₂ first decreased and reached zero point at 4 mg L^{-1} , then reversed as the concentration of HA increased further. As shown in Figs. 1b and 5d, the p-PS-NH₂ showed strong aggregation and reached a maximum hydrodynamic diameter in HA concentration of 10 mg L^{-1} owing to the charge neutralization. It should be noted that different NPs and HA concentrations were used in the aggregation experiments and zeta potential measurements. But the concentration ratios (HA: NPs) are very close to the maximum aggregation point (10: 300) and the charge neutralization point (4: 100). The DLVO profile (Fig. S3d) also indicated that with a slight increase in HA concentration, the net energy barrier reduced, resulting in a large aggregation. However, further increased in the HA concentration led to more negative charges on the NPs surface, and the net energy barrier increased indicating electric repulsion prevailed, and the solution was more stable.

The hydrodynamic diameter of PS in the 10‰ and 30‰ solutions both decreased with increasing HA concentrations (Fig. 5a). The zeta potential of PS in different HA concentrations is presented in Fig. 4b and illustrates that the addition of HA produces more negative charges on the surface of the NP particle, thus the EDL repulsive energy increased and the suspension became more stable. These results were further supported by the DLVO calculation profile (Fig. S3c). The net energy between the particles barrier grew higher as the HA concentration increased. Consequently, the NP particles stabilized, which is consistent with the aggregation experiment and the zeta potential measurements. These results imply that HA increased the NPs surface potentials and produced net energy barriers between particles, thus stabilizing the NPs.

The relatively stabilizing effect of HA reduced with the increased salinity, as illustrated in Fig. 5b. The results are realistically significant, in that areas of higher salinity, despite the presence of DOM, NPs form larger aggregates and become less stable. Interactions between salinity and HA do exist, and they influence environmental aggregation behaviour jointly.

3.4. Effect of HA on the acute toxicity of NPs to *Daphnia magna*

Surface characteristics and aggregation influence not only the distribution of NPs but also their toxicity. The 48 h acute toxicity of NPs on *Daphnia magna* was tested. All the *Daphnia magna* exposed to control solution were alive, indicating that the ethanol used to prepare the PS suspensions did not affect the toxicity. Considering the concept of “acute toxicity”, the NPs concentration used was higher than the environmental concentration to induce evident toxicity to *Daphnia magna*. The results (Fig. 6a) showed that the survival rate of *Daphnia magna* exposed to 20 mg L^{-1} PS, n-PS-NH₂, PS-COOH and p-PS-NH₂ was $15 \pm 10\%$, $20 \pm 16\%$, $30 \pm 11\%$ and $95 \pm 10\%$, demonstrating a different effect of surface charge on toxicity. This result is inconsistent with previous research which found that positively charged NPs were more toxic than negatively charged ones (Manfra et al., 2017; Nomura et al., 2013). As for yeast cells, positively charged NPs neutralized the surface charge on the cell membrane thus induced cell death. However, our study used *Daphnia magna* as the test organism, which is an arthropod crustacean with a protective chitinous exoskeleton, so we assumed that the chitinous exoskeleton could prevent the direct contact of PS from the skin cell of *Daphnia magna*. Nevertheless, more investigation should be conducted to reveal the interaction between NPs and *Daphnia magna*.

In addition, we tested the toxicity of NPs when HA was present. As shown in Fig. 6b and c, the presence of HA significantly reduced the toxicity of PS and p-PS-NH₂ on *Daphnia magna*. In the exposure solution without HA, the survival rate was 15 ± 10% and 45 ± 19% in the solution with 20 mg L⁻¹ PS and 30 mg L⁻¹ p-PS-NH₂, respectively. In this experiment, we observed that *Daphnia magna* was partly or completely wrapped by the NPs floccule, and this heavy burden caused them to slow their movement and consequently led to exhaustion. During the test, a macroscopic cluster of floccule trailed was found on *Daphnia magna* exposed to NPs, and the phenomenon was alleviated when HA was added. Additionally, the survival rate was significantly increased (Fig. 6b and c). This effective alleviation of toxicity may be attributed to the adsorption of HA onto PS and p-PS-NH₂, contributing negative charges and stabilizing the suspension as mentioned above. This reduced the NPs aggregation onto the *Daphnia magna* and consequently reduced their entanglement and body burden. Furthermore, once being ingested, the HA absorbed onto the NPs surface can then separate the NPs from the intestinal tissue and cells in the *Daphnia magna*, thus cutting off the contact. Meanwhile, the hydrophobic NPs may tend to adsorb on the cells, while the HA makes them more hydrophilic and reduces this adsorption.

4. Conclusion

HA is a major component of DOM which is ubiquitous in the aquatic environment. On the other hand, salinity is a distinctive feature in estuaries. Our study provided insight into the effect of HA and salinity on the aggregation behaviour and toxicity of NPs. The observations indicated that the stability, transport and toxicity of NPs in aquatic systems were highly dependent on the environmental factors. Salinity accelerated the NPs aggregation, while HA mainly exerted a stabilizing effect on the three negatively charged NPs but played a different role in the aggregation process of p-PS-NH₂. The acute toxicity of NPs on *Daphnia magna* was also influenced by the presence of HA which could decrease the aggregation of NPs and reduce the entanglement and body burden to *Daphnia magna*. Our findings confirmed that HA and salinity have a profound impact on the ecological consequences of NPs, and laid a foundation for the prediction of stability, transport and toxicity of NPs in different environmental conditions. On the other hand, additional studies are required to in-depth understand the interaction between NPs and environmental factors in the aqueous environment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2018.11.055>.

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