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2019

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### How to cite

SAAVEDRA VARGAS, Juan, STOLL, Serge, SLAVEYKOVA, Vera. Influence of nanoplastic surface charge on eco-corona formation, aggregation and toxicity to freshwater zooplankton. In: Environmental Pollution, 2019, vol. 252, p. 715–722. doi: 10.1016/j.envpol.2019.05.135

This publication URL: <https://archive-ouverte.unige.ch/unige:127441>

Publication DOI: [10.1016/j.envpol.2019.05.135](https://doi.org/10.1016/j.envpol.2019.05.135)

**Influence of nanoplastic surface charge on eco-corona  
formation, aggregation and toxicity to freshwater  
zooplankton**

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## Abstract

The concerns about possible environmental implications of nano- and micro- plastics are continuously raising. Hence, the comprehensive understanding of their behaviour, bioaccumulation and toxicity potential is required. Nevertheless, systematic studies on their fate and possible effects in freshwaters, as well as the influence of particle-specific and environmental factors on their behaviour and impacts are still missing. The aims of the present study are thus two-fold: (i) to examine the role of the surface charge on nanoplastic stability and acute effects to freshwater zooplankton; (ii) to decipher the influence of the refractory natural organic matter (NOM) on the nanoplastic fate and effects. Amidine and carboxyl-stabilized polystyrene (PS) spheres of 200 nm diameter characterized by opposite primary surface charges and neutral buoyancy were selected as model nanoplastics. The results demonstrated that the surface functionalization of the polystyrene nanoplastics controls their aggregation behaviour. Alginate or Suwannee River humic acid (SRHA) modified significantly the surface charge of positively charged amidine nanoplastic and the aggregation state, while had no significant influence on the negatively charged carboxyl nanoplastic. Both amidine and carboxyl nanoplastics were ingested by the zooplankton and concentrated mainly in the gut of water flea *Daphnia magna* and larvae *Thamnocephalus platyurus*, and the stomach of rotifer *Brachionus calyciflorus*. Amidine nanoplastic was more toxic than carboxyl one. The toxicity decreased in the order *D. magna* (48 h - immobilization) > *B. calyciflorus* (24 h - lethality) > *T. platyurus* (24 h - lethality). Alginate or SRHA reduced significantly the toxicity of both amidine and carboxyl PS nanoplastics to the studied zooplankton representatives. The implications of this laboratory study findings to natural environment were discussed.

**Keywords:** Nanoplastics, NOM, amidine, carboxyl, ecotoxicity, *D. magna*, *B. calyciflorus* and *T. platyurus*.

**Capsule:** Natural organic matter affects the surface charge of model nanoplastics and decreases their toxicity to zooplankton in a way dependent on the nanoplastic primary functionalization

## 1. Introduction

Worldwide plastic production is continuously growing making plastics pollution a global environmental threat (Au et al., 2017; Avio et al., 2017; Barboza and Gimenez, 2015; Galloway et al., 2017). As a consequence the abundance of the (sub)micro-sized plastic materials (<5mm) in the aquatic environment is significantly raising, as well as, the concerns about their environmental implications (Alimi et al., 2018; Thompson et al., 2009). Indeed, there are multiple and growing evidences regarding possible toxicity of nano- and micro-plastics to aquatic organisms, including algae, ciliates, invertebrates, crustaceans and fish (Chae and An, 2017). It was shown that nano- and micro- plastics accumulate in aquatic organisms (Galloway et al., 2017; Green et al., 2017) and could cause adverse effects including growth inhibition (Sjollema et al., 2016), immobilization (Rehse et al., 2016), behavioural disorders (Sussarellu et al., 2016), reproductive dysfunction (Canesi et al., 2015) and reduced viability (Nasser and Lynch, 2016). However a controversy exists in the literature dealing with the fate and biological impacts of nano- and micro- plastics, as well as important methodological issues as highlighted recently (Karami, 2017).

Opposite to the important advancements in the understanding of the fate and impact of nano- and micro- plastics in marine environment (Galloway et al., 2017; Galloway and Lewis, 2016), similar studies in freshwater environments or with freshwater species are rather limited (Alimi et al., 2018; Eerkes-Medrano et al., 2015; Triebskorn et al., 2019; Wagner et al., 2014). Polyethylene particles (1 – 4  $\mu\text{m}$ ) induced mobilization of water flea *Daphnia magna*, whereas particles of 90 – 106  $\mu\text{m}$  size had no significant effects on the same organism (Rehse et al., 2016). Hence, the comprehensive understanding of their behaviour, bioaccumulation and toxicity potential in freshwaters is highly sought.

The surface properties of the nano- and micro- plastics, including surface functionalization and charge, were recognised to play a determinant role in their ecological and ecotoxicological outcomes (Galloway et al., 2017). Limited literature concerning nano- and micro- plastics revealed that amidine PS particles (presumably positively charged) were toxic to sea urchin embryo (Della Torre et al., 2014) and water flea *D. magna* (Nasser and Lynch, 2016) opposite to the carboxyl one. However, negatively-charged carboxyl PS particles immobilized *D. magna* stronger than non-functionalized one (Kim et al., 2017). Nevertheless, the role of the surface functionalization and charge on the behaviour of nano- and micro- plastics in freshwater is largely unexplored.

In the aquatic environments, the surface of nano- and microplastics is rapidly coated by different components of the natural organic matter (NOM) such as humic substances, extracellular polymeric substances, proteins etc. (Alimi et al., 2018; Galloway et al., 2017). The formed eco-corona thus modulated their bioreactivity and potential impacts (Pulido-Reyes et al., 2017). However, the consequences of the eco-corona on the possible toxicological consequences of nano- and microplastics exposure are not studied systematically.

The primary goal of this study is to improve the current understanding on the behaviour and effects of nanoplastics in freshwater environment. The specific aims were: (i) to examine the role of the particle surface functionalization and charge on their stability and aggregation, and on the acute effects to freshwater zooplankton; (ii) to decipher the role of freshwater NOM on the nanoplastic fate and effects. To this end, we choose to study amidine and

carboxyl-stabilized polystyrene nanospheres as model nanoplastics. Water flea *D. magna*, rotifer *Brachionus calyciflorus* and larvae *Thamnocephalus platyurus*, were selected as representative of freshwater zooplankton. Humic acid and alginate were used as models of the two major types of refractory NOM typically found in freshwater ecosystems.

We hypothesised that: (i) particle aggregation, bio-reactivity and ecotoxicological outcome will depend on their primary surface functionalization and charge; (ii) NOM found in freshwaters will interact with the particles and will form eco-corona, which will affect both their aggregation and interactions with bio-interfaces; (iii) as a consequence NOM will reduce the particle toxicity and protect the organisms from nanoplastic-induced stress. We also assumed that the nanosphere behaviour and induced effects differ from those of natural particles given their significant physical and chemical difference in particle morphology, heterogeneity, surface charge (Oriekhova and Stoll, 2018). As a result, the residence times, the interactions with freshwater zooplankton, NOM, etc. and thus ecological outcome could be significantly different.

## **2. Materials and methods**

### **2.1 Nanoplastics, natural organic matter, test organisms and experimental setup**

Amidine (PS(-CNH<sub>2</sub>NH<sub>2</sub><sup>+</sup>), positive) and carboxyl (PS(-COO<sup>-</sup>), negative) stabilized hydrophobic polystyrene nanospheres with a 200 nm diameter, were selected to examine the influence of the surface charge on eco-corona formation, aggregation and toxicity. Surfactant-free aqueous stock suspensions (4% w/v) of 43 and 41 g/L were purchased from Invitrogen (Thermofisher scientific, USA). Primary characteristics of the nanospheres are given in **Table S1**. Particle suspensions were homogeneous and composed of monodispersed

units having identical specific surface areas. The density of the PS nanospheres ( $1.055 \text{ g cm}^{-3}$  at  $20^\circ\text{C}$ ) is comparable with the density of freshwaters, which classifies them as a neutrally buoyant.

To get an insight into the role of NOM on particle aggregation and toxicity, two representative environmental compounds, i.e. humic substances and polysaccharide chains, were selected. A standard humic acid isolated from the Suwannee River, SRHA, Georgia, was purchased from the International Humic Substances Society (Colorado School of Mines, Golden, CO). The SRHA powder was dissolved in ultrapure water to obtain stock solutions of  $1 \text{ g L}^{-1}$ . Intermediate solutions of  $100 \text{ mg L}^{-1}$  were prepared for further dilution. Concerning the polysaccharide chains, alginate isolated from brown alga *Macrocystis pyrifera* was purchased from Sigma-Aldrich Switzerland (A2158). A  $100 \text{ mg L}^{-1}$  stock solution was prepared in ultrapure water and used for further dilution.

The toxicity of the particles was studied on model organisms representative of the low trophic level primary consumers in freshwaters, including zooplankton species *Daphnia magna*, *Brachionus calyciflorus* and *Thamnocephalus platyurus*, which have received little attention until now. They are largely used in ecotoxicological testing due to their sensitivity to different pollutants (Rojíčková-Padrťová et al., 1998). The stability of particles and NOM-coated particles in exposure media was examined prior exposure of the selected organisms.

## **2.2 Characterization of nanoplastic suspensions**

The stability and aggregation behaviour of the model nanoplastics were investigated by considering their surface charge and size variations. More specifically, Zeta potential and Z-

average hydrodynamic diameters were used. Zeta potential measurements were performed to elucidate the aggregation and interaction mechanisms, owing to the fact that they are mainly driven by electrostatic interactions and eco-corona formation, by considering the nanoplastic surface charge modifications due to NOM adsorption and salt effects. Zeta potential measurements allow determining the effective charge at the particle surface and thus can give useful information on the amount of adsorbed NOM as well as charge evolution of the particles with NOM dosage, exposure media properties and ability to form aggregates. Aggregate formation was quantified via determination of the Z-average diameters. For that purpose, PS nanosphere suspensions of 50 mg L<sup>-1</sup> were prepared in the exposure media of different organisms and homogenized by using a vortex with rotational speed of 250 rpm. Zeta potential and Z-average diameters were determined in the absence and presence of 1-5 mg L<sup>-1</sup> SRHA or alginate. The behaviour of the particles was also examined in ultrapure water at different pHs. The Zeta potential and the Z-average diameter of the particles were measured using a Zetasizer Nano (Malvern Instruments, Ltd, UK). The autocorrelation function accumulated at least ten runs for each samples for five parallel measurements with time delay of 5 s. The Stokes-Einstein equation was applied to calculate the hydrodynamic diameter. The electrophoretic mobility was measured using the Doppler method. Then the Smoluchowski equation was used to calculate the corresponding Zeta potential.

### **2.3 Exposure of aquatic organisms to nanoplastics**

The toxicity of amidine and carboxyl PS nanospheres on the three model zooplankton species in the absence and presence of SRHA or alginate were examined by using cyst based-bioassays. DAPHTOXKIT F-magna for *D. magna*, acute ROTOXKIT F for *B. calyciflorus* and THAMNOTOXKIT F for *T. platyurus* (Microbiotests, Ghent, Belgium) were employed.

The selected cyst acute toxicity tests adhere to OECD guideline 202 and ISO 6341 for *D. magna*, ISO 14380 for *T. platyurus* and ASTM standard guide E 1440-91 for *B. calyciflorus*. They were chosen as a rapid and less labour-intensive alternative of the culture-based conventional tests with neonates, which do not require stock culturing of test organisms. Comparative research revealed similar sensitivity of the cyst-based kits for *D. magna* (Persoone et al., 1994), *B. calyciflorus* (Janssen et al., 1994) and *T. platyurus* (Centeno et al., 1995) with standard toxicity tests. The organisms were exposed to variable concentrations of amidine and carboxyl PS nanospheres increasing from 10 to 400 mg L<sup>-1</sup>. Standard operation procedures recommended by the kit provider were followed as further detailed in the SI. In all cases, at least three independent assays were conducted. The test was taken as valid if the immobilization of control essays did not exceed 10%. Unexposed control groups were run for all experiments. The concentrations of nanoplastics inducing immobilization or mortality in 50% of the exposed organisms (EC<sub>50</sub>) were calculated using a *Probit* analysis which is a special regression model of binomial response variable (Vanewijk and Hoekstra, 1993). The accumulation of the particles in the organisms was followed at the end of the exposure by using an optical microscope (BX61, Olympus, Volketswil, Switzerland).

#### **2.4 Data treatment**

Data treatment was performed using OriginPro 8 (Data Analysis and Graphic Software, Northampton, Massachusetts, USA). One way ANOVA was used to compare the treatments with different nanoplastics and zooplankton. When the null hypothesis, which refers that there is no significant difference among the studied groups ( $p > 0.05$ ), was rejected, the Tukey's Honest Significant Difference Test was applied to determine the individual significant difference from a set of means.

### 3. Results and discussion

#### 3.1 Behaviour of nanoplastics in the different exposure media

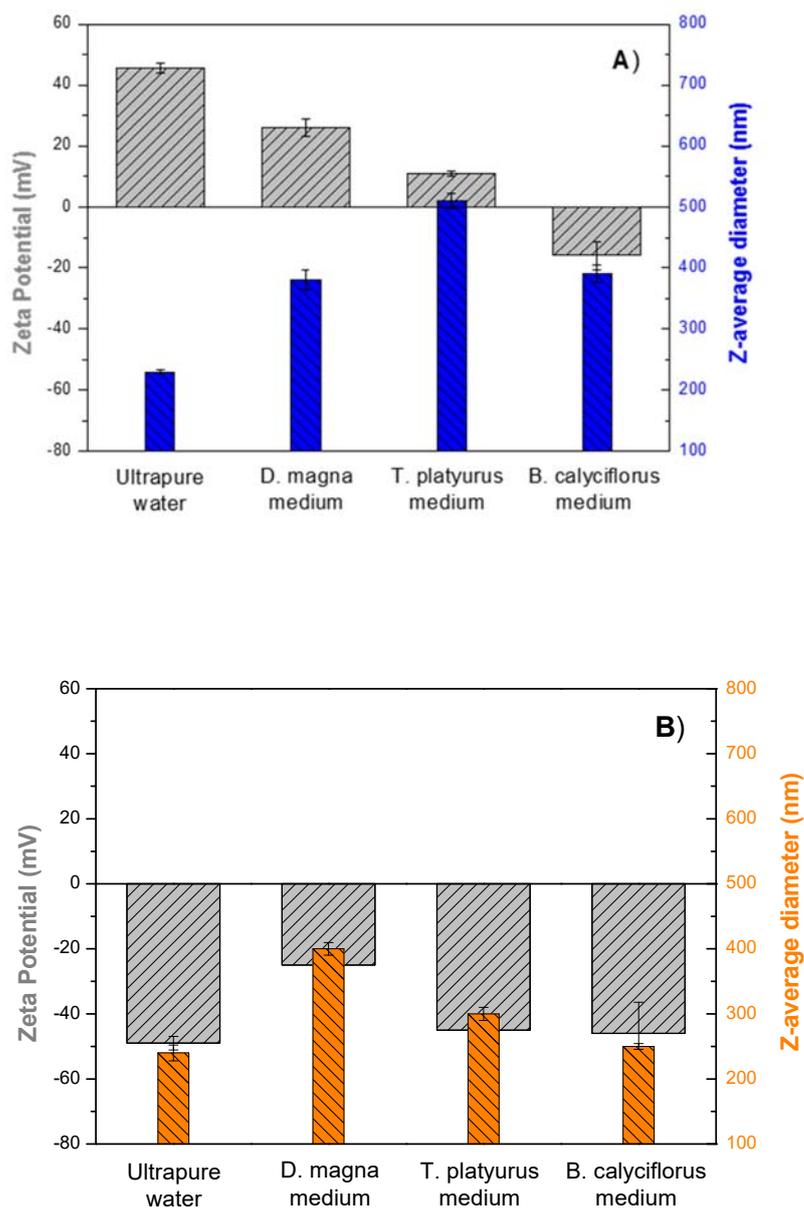
Before considering the behaviour of nanospheres in the exposure media, Zeta potential and Z-average diameters were determined in ultrapure water at different pH values. Amidine nanoplastics exhibited positive Zeta potential values around +50 mV within the pH range from 3.0 to 11.0. Due to electrostatic repulsions, amidine PD nanospheres were stable and homogeneously dispersed in particular at pH of 8.0, representative of the organism exposure media pH (**Fig. S1A**). Under such condition, the Z-average diameter was equal to 226.4 d.nm ( $\pm 8.6$ ). Carboxyl PS nanospheres were negatively charged for pH from 5.0 to 11.0, with Zeta potential values around -50 mV and with Z-average diameters equal to 220.1 d.nm ( $\pm 9.1$ ) at pH 8.0 (**Fig. S1B**).

Eco-corona formation in presence of alginate or SRHA was investigated at pH 8.0 and variable NOM concentration. Both alginate and SRHA, which are negatively charged, interact rapidly with the positive amidine PS nanospheres, resulting in a significant decrease of Zeta potential (**Fig. S2**). Addition of the 1 mg L<sup>-1</sup> of alginate or SRHA to amidine PS nanospheres resulted in reversing of the Zeta potential from + 50 mV to -39.0 ( $\pm 0.5$ ) mV and -47.0 ( $\pm 0.9$ ) mV, respectively. A rapid increase of Z-average diameter was observed with values equal to 924.0 ( $\pm 94.6$ ) and 670.6 d.nm ( $\pm 21.8$ ), respectively. It should be noted that alginate, due to its structural conformation (long linear chains), is leading to the formation of larger aggregates in comparison with humic acid having a more compact structure. Amidine charge neutralization, which corresponds to the isoelectric point (IEP), was observed for an alginate or SRHA concentration equal to 0.39 and 0.47 mg L<sup>-1</sup> respectively. Zeta potential of the amidine PS nanospheres was unchanged at NOM

concentrations higher than 1 mg L<sup>-1</sup>. Carboxyl PS nanospheres were stable in presence of NOM. No significant change of their Zeta potential was observed in presence of SRHA or alginate. Z-average diameters remained constant (**Fig. S1B**) indicating the absence of aggregation due to the electrostatic repulsions between NOM and carboxyl nanospheres. These results are in good agreement with a previous study which considered PS sulfate nanospheres in presence of alginate at variable concentration (Ramirez et al., 2016). Ultrapure water was used as a reference system in which at pH 8.0 the respective Zeta potential values in the presence of alginate and humic acid were found equal to -45.3 (± 2.1) mV and -59.4 (± 3.1) mV, respectively. Overall, based on the model nanoplastics sizes and electrical surface charges measurements, the comparable density with that of freshwaters and their neutral buoyancy the PS particles are expected to remain suspended and have a potential to affect the zooplankton species in the water column.

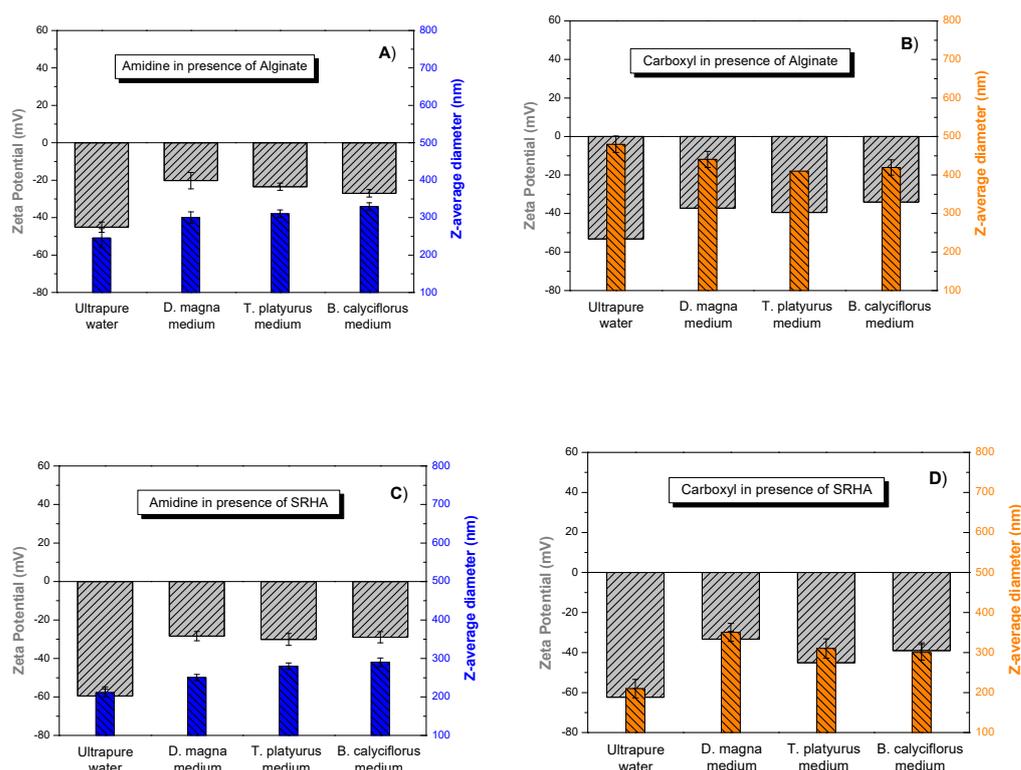
The behaviour of the model nanoplastics was also investigated in the different exposure media. Zeta potential of the amidine PS nanospheres was reduced in *D. magna* and *T. platyurus* culture media (**Fig. 1A**) due ionic strength effects which are expected to compress the particle electric double layer and therefore decrease the Zeta potential. In the *B. calyciflorus* medium, Zeta potential had a negative value of -16.4 mV (± 4.3) indicating possible specific adsorption of anions in addition to electrical double layer compression. In good agreement with the different Zeta potential values, a significant increase of Z-average diameter was observed in all the exposure media in comparison with ultrapure water indicating the formation of aggregates. For carboxyl PS nanospheres (**Fig. 1B**), limited change of the Zeta potential was found if comparison is made with ultrapure water and

aggregation occurred in most exposure media, especially in *D. magna* medium due to screening effects or specific adsorption of cations.



**Figure 1.** Zeta potential and Z-average diameter of (A) amidine and (B) carboxyl PS nanosphere suspensions in different exposure media (Ultrapure water, *D. magna* medium, *T. platyurus* medium and *B. calyciflorus* medium) at concentration of  $2 \text{ mg L}^{-1}$  and pH 8.0.

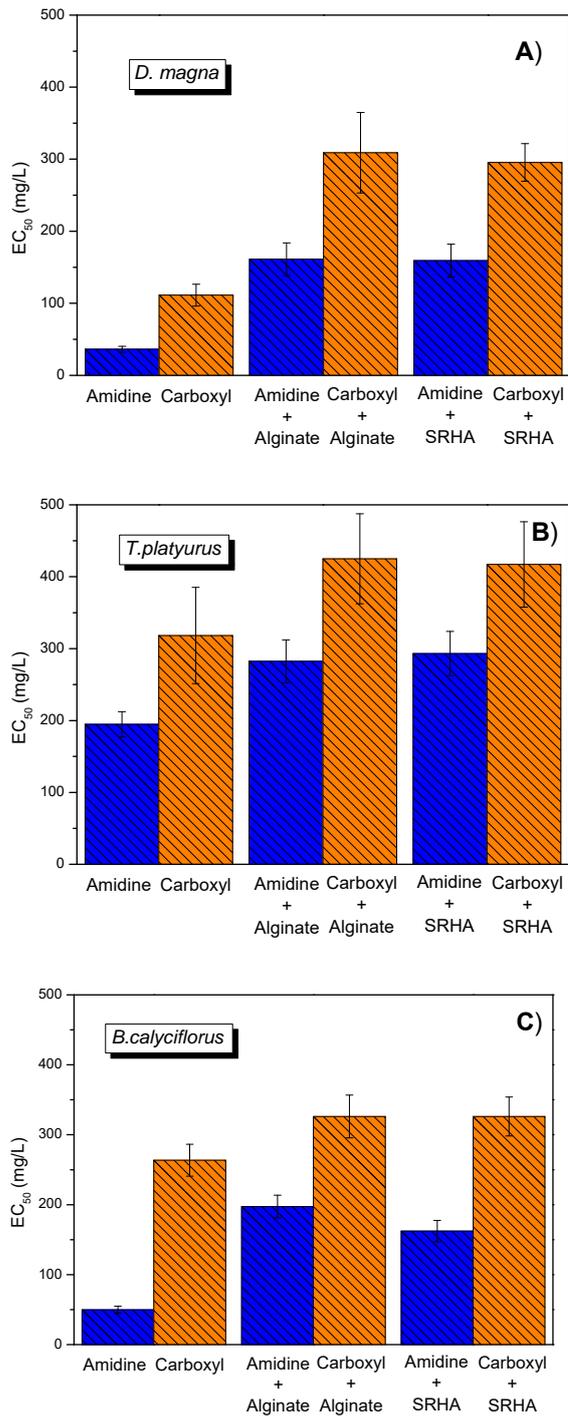
The behaviour of amidine and carboxyl PS nanospheres in presence of 2 mg L<sup>-1</sup> alginate or SRHA was further investigated in the different exposure media. When amidine PS nanospheres were mixed with alginate or SRHA in the different exposure media (Fig.2 A, C), Zeta potential decreased significantly (towards less negative values) and aggregation was limited by the presence of NOM, hence indicating NOM stabilizing effect due to the formation of eco-corona. Regarding carboxyl PS nanoplastics in presence of alginate or SRHA, no significant change of the Zeta potential were observed (Fig. 2 B, C). However, the aggregation was more significant in presence of alginate due to bridging effects or presence of alginate aggregates.



**Figure 2.** Zeta potential and Z-average particle diameter of (A, C) amidine and (B, D) carboxyl PS nanospheres (5mg L<sup>-1</sup>) in different exposure media (Ultrapure water, *D. magna*, *T. platyurus* and *B. calyciflorus*) in the presence of 2 mg L<sup>-1</sup> alginate (A, B) or SRHA (C, D), pH 8.0.

### 3.2 Effect of amidine and carboxyl nanoplastics to aquatic organisms

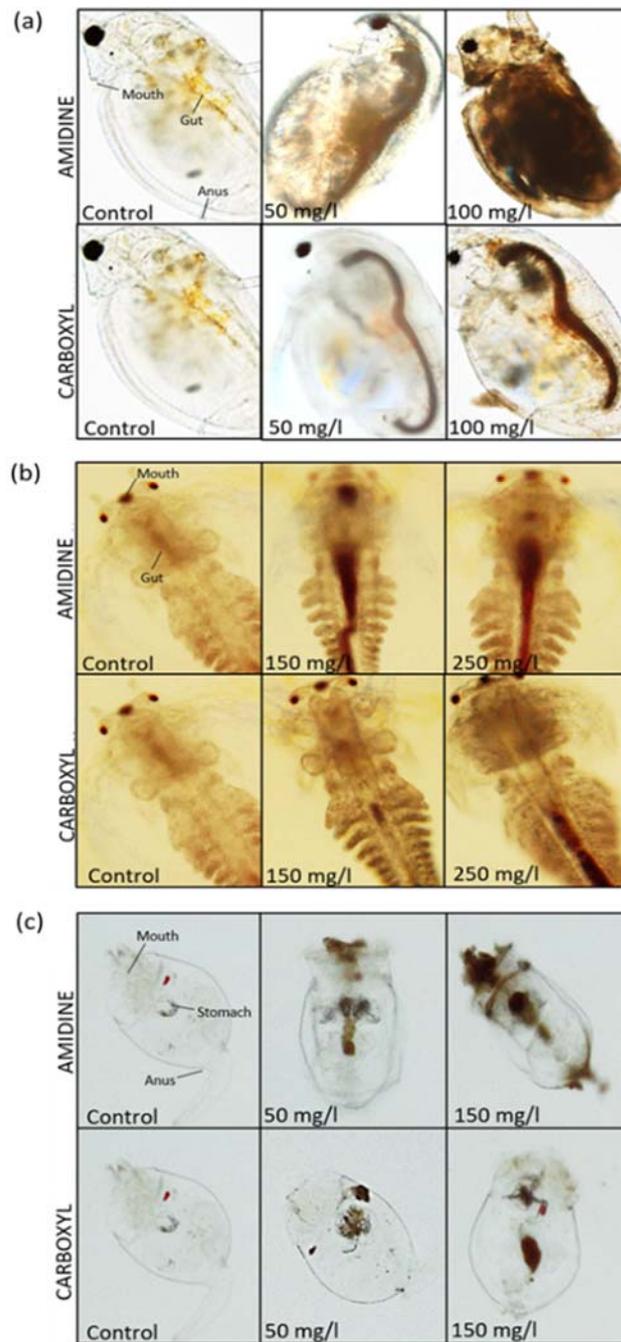
The results of the comparative acute toxicity tests on *D. magna*, *T. platyurus* and *B. calyciflorus* indicated that amidine PS nanospheres exhibit stronger negative effect than the carboxyl one. The percentages of 48 h - immobilized *D. magna*, 24 h - dead *T. platyurus* or *B. calyciflorus* increased significantly with particle concentrations (**Fig. S3**) over the tested concentration range. The EC<sub>50</sub> for amidine PS nanoplastics were lower as compared with those obtained for carboxyl PS nanospheres, showing its higher toxicity. The slopes of dose-response's curves were steeper, suggesting a stronger response of the species to amidine PS nanosphere exposure. For example, for the most sensitive organism, *D. magna*, the positively charged amidine PS nanospheres were approximately 3 times more toxic (EC<sub>50</sub> = 36.2 ± 4.1 mg L<sup>-1</sup>) than carboxyl PS nanospheres (EC<sub>50</sub> = 111.4 ± 15.2 mg L<sup>-1</sup>). This observation could be related with different type of functional groups and surface charge in the studied nanoplastics and is consistent with the positive values of the Zeta potential measured for amidine PS nanoplastics in *D. magna* medium. Indeed, positive charges were shown to enable particles to interact easily with negatively charged cell membranes (Nel et al., 2006). The above findings are in line with published literature revealing severe detrimental effects of amidine PS particles to sea urchin embryo and lack of toxicity of carboxyl PS particles (Della Torre et al., 2014) and to *D. magna* (Nasser and Lynch, 2016). However, no data for the surface charge or Zeta potential were provided in this study to enable further conclusion on their role as toxicity driver. It should be noted that our results diverge with other studies showing that negatively charged carboxyl PS particles induced about twice stronger immobilization of *D. magna* than non-functionalized PS (Kim et al., 2017). Exposure to 1 μm - polyethylene particles induced 48 h immobilization in less than 10% in *D. magna*, while after 96 h EC<sub>50</sub> value of 57.43 mg L<sup>-1</sup> were found (Rehse et al., 2016).



**Figure 3.** Acute EC50 values for zooplankton species exposed to amidine and carboxyl PS nanospheres (A) *D. magna* (48 h - immobilization test), (B) *T. platyurus* (24 h - lethality test), (C) *B. calyciflorus* (24 h - lethality test). The concentration of alginate and humic acid is equal to 2 mg L<sup>-1</sup>, pH=8.0.

The acute EC<sub>50</sub> (Fig. 3, Table S3) and toxicity slopes in the dose-response curves (Fig. S3) revealed that the sensitivity of the tested zooplankton species towards both functionalized PS particles decreased in the order *D. magna* (48 h - immobilization) > *B. calyciflorus* (24 h - lethality) > *T. platyurus* (24 h - lethality). This species sensitivity trend is consistent with several studies with nano- and micro- plastics of various size and surface functionalization showing that upon acute exposure *D. magna* was stronger affected than *T. platyurus* (Casado et al., 2013; Naha et al., 2009). Indeed the polyethyleneamine (PEA) PS nanoplastics of 55 and 110 nm nominal size induced 48 h - immobilization of *D. magna* in concentrations, which were about 6 times lower than those inducing 24 h - lethality effect in *T. platyurus* (Casado et al., 2013). Similarly *D. magna* was more sensitive than *T. platyurus* during acute exposure to poly N-isopropylacrylamide and N-isopropylacrylamide/N-tert-butylacrylamide copolymer nanoparticles of 50 – 70 nm (Naha et al., 2009).

Overall, a dose-dependent effect for three zooplankton species were observed during exposure to 200 nm amidine functionalized PS nanoplastics, consistently showing greater negative effects and with *D. magna* being most sensitive species. The acute EC<sub>50</sub> values obtained in the present study, were within the range for variety of nano- and micro- plastics reported for water flea *D. magna* (Booth et al., 2016; Casado et al., 2013; Naha et al., 2009; Rehse et al., 2016; Rist et al., 2017), and branchiopod *T. platyurus* (Casado et al., 2013; Naha et al., 2009). No similar results are available for acute exposure of rotifer *B. calyciflorus* to enable comparison. Based on the results of the present study amidine PS nanoplastics can be classified as “harmful” for *D. magna* and *B. calyciflorus* and, carboxyl for *D. magna* according EU-Directive 93/67/EEC. Both PS particles can be considered as “Not Classified” with respect to *T. platyurus*.



**Figure 4.** Accumulation of amidine and carboxyl polystyrene nanospheres in different concentrations to (A) water flea *D. magna*, (B) larvae *T. platyurus* and (C) rotifer *B. calyciflorus*.

The addition of 2 mg L<sup>-1</sup> alginate and SRHA to carboxyl or amidine PS nanoplastics suspensions in the exposure media resulted in a substantial decrease of the negative effect of these particles to all three organisms, as revealed by much higher EC<sub>50</sub> values for *D. magna*, *B. calyciflorus* and *T. platyurus* (**Fig. 4** and **Table S3**). These observations are in agreement with the significant reduction of the Zeta potential of the positively charged amidine PS nanoplastics, which in the presence of SRHA or alginate became negative. Such observation is not surprising since NOM of different origin was shown to play a protective role to many aquatic organisms exposed to various pollutants including nanoparticles (Alimi et al., 2018; Slaveykova and Wilkinson, 2005; von Moos et al., 2014). Furthermore, the EC<sub>50</sub> values for the three organisms were comparable in the presence of SRHA or alginate, which suggests that the nature and structure of the formed eco-corona has a secondary importance in modulation of particle bio-reactivity. Nevertheless, the surface functionalization of “as produced” PS nanoplastics seems to play a significant role in eco-corona formation by SRHA or alginate and the effects to biota since the amidine PS nanoplastics induced stronger effects than carboxyl one even in the presence of NOM. By further exploring the possible link between the PS nanoplastics – induced acute effects in the three zooplankton species and their characteristics (Zeta potential and Z-average diameters) in the exposure medium, a good correlation was found between the intensity of the induced effect and the Zeta potential in the exposure media in the absence of NOM. This correlation could be linked to the fact that the amidine PS nanoplastics are positively charged, feature that makes easy the interaction with the microorganisms, which are, by default, negatively charged (Nel et al., 2006). On the other hand, the carboxyl PS nanospheres, that have the same negative electric charge as the microorganisms, generate a repulsion force once ingested, reducing the interaction between this pollutant and the organism. It is also in line with the existing research on the engineered

nanoparticles (Powers et al., 2007) and the limited number of studies showing that surface chemistry of plastic nanoparticles may play an important role in modulating particle toxicity (Booth et al., 2016; Casado et al., 2013; Della Torre et al., 2014; Naha et al., 2009). For example a correlation between the ecotoxicological response of algae, daphnids and bacterium *Vibrio fisheri* and Zeta potential values was reported for poly-N-isopropylacrylamide and N-isopropylacrylamide/N-tert-butylacrylamide copolymer nanoparticles of 50–70 nm in the respective exposure media (Naha et al., 2009).

### **3.3 Bioaccumulation of nanoplastics by zooplankton**

The microscopy observations revealed that the accumulation of the nanoplastics depended on both the organism and the PS surface functionalization (**Fig. 4**). Opposite to the transparent body of unexposed control organisms, an accumulation of the dark-coloured material in zooplankton was observed upon exposure to PS nanospheres, showing that all the studied organisms ingested nanospheres of 200 nm and their aggregates. Particles concentrated mainly in the gut of *D. magna* and *T. platyurus*, and the stomach of *B. calyciflorus*. This observation is consistent with the existing literature for *D. magna* demonstrating the ability of the water flea to ingest micro- and nano- plastics of different (20 nm to 5 µm) sizes and shapes (spheres, fragments and fibres) (Jemec et al., 2016; Ogonowski et al., 2016; Rosenkranz et al., 2009). The carboxylated polystyrene beads rapidly accumulate in the gastrointestinal tract of *D. magna* adults and neonates, and the oil storage droplets, suggesting that they have crossed the gut's epithelial barrier (Rosenkranz et al., 2009). Furthermore, a protein corona formed on the nanospheres (88 nm initial size) accumulated by *D. magna* resulted in a higher retention of corona-coated amidine and

carboxyl polystyrene particles (Nasser and Lynch, 2016). No similar data for *T. platyurus* and *B. calyciflorus* are available to allow comparison.

In the present study, the amount of the amidine and carboxyl PS nanospheres in the organism body increased with their concentration in the exposure medium, suggesting dose-dependent accumulation. This is opposite to the previous study (Rehse et al., 2016) showing no concentration dependence of the particle loads in *D. magna* ingesting 1  $\mu\text{m}$  PE particles. Here, the accumulation of amidine PS nanospheres was much more pronounced than that of carboxyl one in all organisms demonstrating the important role of the particle surface characteristics for their binding onto and into the organisms. This finding is consistent with the previous study (Della Torre et al., 2014) showing a dependence of the nanoplastics accumulation on their surface characteristics. Carboxyl functionalized polystyrenes accumulated inside the digestive tract of sea urchin embryo (Della Torre et al., 2014). 8  $\mu\text{m}$  polystyrenes particles with  $-\text{NH}_2$  and  $-\text{COOH}$  functionalization build up in different locations in the grab gills, which probably is related to their different binding to biota (Watts et al., 2016). In the present study large particle load of amidine PS nanospheres was observed in *D. magna* which whole surface was covered at exposure concentrations larger than 100  $\text{mg L}^{-1}$  (**Fig. 4**). Such a high accumulation of amidine PS nanospheres into and onto body of *D. magna* could be explained with their positive charge in the absence of NOM (**Fig. 1**), as well as longer exposure time (48 h). In the presence of NOM (data not shown), the amount of the accumulated amidine and carboxyl PS particles decreased significantly and in agreements with the results of the acute toxicity tests. Indeed an eco-corona represents what the organism sees (Pulido-Reyes et al., 2017) and plays a central role in modulation of the nano- and micro- particle biological reactivity and the responses in living organisms.

Overall the present study revealed that the surface functionalization of the “as produced” nanoplastics determined their stability and toxicity to zooplankton in freshwater environment. The results were obtained under well-controlled conditions employing nanoplastic concentrations much higher than those found in the freshwaters (Faure et al., 2015). Nevertheless, the results have important environmental implications for the improving the understanding on the behaviour of the neutrally buoyant nanoplastics in freshwaters. More specifically, the PS nanospheres were well dispersed in the medium and the formation of NOM eco-corona led to stabilization of their suspensions. In natural environment where the nanoplastics concentrations are much lower, the formation of aggregates will be even less relevant, so they will remain suspended in the water column and thus interact with the organisms present in the water column. The toxicity effects here were observed at nanoplastics concentrations that are several orders of magnitude higher than those currently found in natural waters. However, there are few studies on freshwater system and the types and fates of nanoplastics are still not well-understood as well as their exact concentrations to determine. In addition, the river basins were suggested to be the main exporters of micro- and nano- plastics from the continent to the marine environment, thus the concentrations of microplastics (and nanoplastics) greater in freshwater systems are expected due to the retention capacity of enclosed systems as compared with marine systems, which are semi-enclosed or open (Koelmans et al., 2015). Therefore, an urgent attention on the nanoplastic in freshwater environment is required to further understand their behaviour and possible environmental risks. The present study also revealed that NOM reduced the toxicity of the PS nanospheres to zooplankton. These finding imply that at comparable nanoplastics

concentrations, the potential ecotoxicological impact would be more significant in the freshwater with lower NOM contents as compared with humic substances - rich ecosystems.

#### **4. Conclusion**

The present study demonstrated that the surface functionalization of the polystyrene nanoplastics controls their aggregation behaviour. NOM (alginate and SRHA) stabilized the positively charged amidine PS nanoplastics as observed by the significant decrease of Zeta potentials and Z-average diameters, while had no significant influence on the negatively charged amidine PS nanoplastics. Both types of PS nanoplastics were ingested by the zooplankton and concentrated mainly in the gut of *D. magna* and *T. platyurus*, and the stomach of *B. calyciflorus*. Amidine nanoplastics were more toxic than carboxyl nanoplastics for the three zooplankton species. The toxicity decreased in the order *D. magna* (48 h - immobilization) > *B. calyciflorus* (24 h - lethality) > *T. platyurus* (24 h - lethality). Alginate or SRHA formed an eco-corona on the PS nanoplastics and significantly reduced their acute toxicity. The findings of the present study revealed the potential of the nanoplastics to affect freshwater crustaceans, as well as the importance of the particle surface charge and eco-corona in particle-biota interactions and in their stability in freshwater systems.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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