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## Surface functionalization determines behavior of nanoplastic solutions in model aquatic environments

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### Abstract :

Plastic debris are classified as a function of their size and recently a new class was proposed, the nanoplastics. Nano-sized plastics have a much greater surface area to volume ratio than larger particles, which increases their reactivity in aquatic environment, making them potentially more toxic. Only little information is available about their behavior whereas it crucially influences their toxicity. Here, we used dynamic light scattering (DLS) to explore the influence of environmental factors (fresh- and saltwater, dissolved organic matter) on the behavior (surface charge and aggregation state) of three different nano-polystyrene beads (50 nm), with (i) no surface functionalization (plain), (ii) a carboxylic or (iii) an amine functionalization. Overall, the positive amine particles were very mildly affected by changes in environmental factors with no effect of the salinity gradient (from 0 to 653 mM) and of a range 1–30 µg.L<sup>-1</sup> and 1–10 µg.L<sup>-1</sup> of organic matter in artificial seawater and ultrapure water, respectively. These observations are supposedly linked to a coating specificity leading to repulsive mechanisms. In contrast, the stability of the negatively charged carboxylic and plain nanobeads was lost under an increasing ionic strength, resulting in homo-aggregation (up to 10 µm). The increase in organic matter content had negligible effect on these two nanobeads. Analysis performed over several days demonstrated that nanoplastics formed evolving dynamic structures detected mainly with an increase of the homo-aggregation level. Thus, surface properties of given polymers/particles are expected to influence their fate in complex and dynamic aquatic environments.

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## Highlights

► The behavior of different nanopolystyrene beads was investigated by dynamic light scattering. ► Surface functionalization affects the behavior of nanopolystyrene beads. ► Carboxylate and plain nanopolystyrene beads formed microscale aggregates in seawater. ► Organic matter had negligible effect on all nanoplastics tested. ► Nanoplastics formed evolving dynamic structure over time.

**Keywords** : Nanoplastic, Dynamic light scattering, Behavior, Aggregation, Salinity, Organic matter

## 38 **1 Introduction**

39 Owing to the exponential use of plastic items (335 million tons (MT) produced in 2016) by human  
40 societies, their mismanagement after usage is a considerable problem of this century (Galloway et  
41 al., 2017; PlasticsEurope, 2017). Every year, among the 31.9 MT of plastic wastes that are discarded  
42 in environment, between 4.8 and 12.7 MT end up in oceans (Jambeck et al., 2015; Rochman, 2018).  
43 To date, plastic debris are ubiquitous in freshwater and marine systems from rivers to oceans (Cózar  
44 et al., 2014, 2017; Lebreton et al., 2017; Woodall et al., 2014).

45 For about a decade, the research emphasis is laid on the small plastic debris called “microplastics  
46 (MP; < 1 mm)” originating from manufactured beads/pellets/fibers or mostly (> 80%) the  
47 weathering of bigger wastes under environmental conditions (UV light, mechanical degradation,  
48 biodegradation) (Hüffer et al., 2017; Galloway et al., 2017). However, a new class of smaller debris  
49 than MP was proposed lately, the nanoplastics (NP) (Koelmans et al., 2015) which their first report  
50 was argued in the North Atlantic Gyre (Ter Halle et al., 2017). To date, several classifications of NP  
51 were proposed : <20  $\mu\text{m}$  according to the size used to classify nanoplankton (Wagner et al., 2014);  
52 <1 $\mu\text{m}$  owing to the colloidal nature of NP (Gigault et al., 2018); <100 nm in the narrower sense of  
53 the definition of engineered nanomaterials (Mattsson et al., 2015). This last classification is the one  
54 adopted for this present study. Similarly to MP, NP in the oceans can originate from a direct release  
55 from cosmetics (Hernandez et al., 2017), industrial activities (Dubey et al., 2015; Stephens et al.,  
56 2013; Zhang et al., 2012), drugs (Lusher et al., 2017); or from weathering of bigger waste as it was  
57 demonstrated in laboratory under biotic (Dawson et al., 2018) or abiotic conditions (Gigault et al.,  
58 2016; Lambert and Wagner, 2016).

59 Nanoplastics are known to have a higher surface area/volume ratio than MP. Thus, increasing  
60 interactions with persistent organic pollutants (POP) (Liu et al., 2018; Velzeboer et al., 2014) and  
61 biological membranes (Rossi et al., 2014) calling for an accurate description of NP in the context of  
62 the chemical/biological risks in aquatic systems (Koelmans et al., 2016; Paul-Pont et al., 2018).  
63 Although ecotoxicological studies reported higher detrimental effects of NP than MP (*e.g.* Jeong et  
64 al., 2016; Tallec et al., 2018), the behavior of NP (*e.g.* interactions of NP amongst themselves and  
65 other component such as organisms and macromolecules) in experimental environments and even  
66 more in freshwater and marine environments remains largely unknown. However, because the  
67 behavior drives fate and toxicity of nanoparticles (Lowry et al., 2012), it is crucial to fill the gaps  
68 limiting our knowledge in order to understand the toxicity for aquatic life (*e.g.* Paul-Pont et al.,  
69 2018), risks for the balance of ecosystems (Galloway et al., 2017; Mattsson et al., 2015) and up to  
70 human health (Wright and Kelly, 2017). Based on works from other nanomaterials, the behavior of  
71 NP may be driven by three main processes: physical transformations (homo- or hetero-  
72 aggregation); biological transformations (interaction with all components of a biological system  
73 involving oxidation and redox mechanism transforming the surface layer of particle); interaction  
74 with macromolecules (*e.g.* adsorption of polysaccharide, organic matter, protein) leading to the  
75 development of bio- (in organism) or eco-coronas (in environment) (Galloway et al., 2017; Lowry et  
76 al., 2012; Mattsson et al., 2015).

77 A modelling study using various scenarios showed that NP aggregation in freshwater system could  
78 decrease the risk of NP arrival in the oceans (Besseling et al., 2017). Owing to the high diversity of  
79 polymers found in oceans and thus the high diversity of their chemical structures, this model-based  
80 assumption must be compared with experimental data and *in situ* observations when methods will  
81 be developed for NP. Indeed, as reported for other nanomaterials, variations of the chemical

82 surface of the same material affected greatly its behavior in fluids (El Badawy et al., 2010). Also,  
83 nanoparticles' behaviors are known to be impacted by environmental factors (*e.g.* pH, salinity,  
84 organic matter content) (Keller et al., 2010). Thus, the behavior of NP may be highly complex and  
85 their fate can vary from location to location. Furthermore, from our previous study in which we  
86 revealed significant toxicity of NP on oyster fertilization success and embryo-larval development, NP  
87 behavior was hypothesized as the origin of the toxicity variability of three different 50 nm plastic  
88 beads (Tallec et al., 2018). During the redaction of this present work, Cai et al. (2018) investigated  
89 the short-term influence (600 seconds) of environmental factors on behavior of NP and reported a  
90 low incidence of organic matter on the aggregation kinetics of nanopolystyrene (nano-PS) beads  
91 (plain; 100 nm) in media containing various salt (NaCl – 1-100 mM; CaCl<sub>2</sub> – 0.1-15 mM; FeCl<sub>3</sub> –  
92 0.001-1 mM). However, the size and the surface-functionalization which display an important role in  
93 the behavior of particles (Alimi et al., 2018) were not studied and yet of great interest particularly  
94 below 100 nm (Gigault et al., 2018). Here, we performed Dynamic Light Scattering (DLS) analyses  
95 with nanopolystyrene (50 nm) exhibiting different surface functionalization (carboxyl, amine or  
96 none) employed in previous ecotoxicological studies including ours (Della Torre et al., 2014; Jeong  
97 et al., 2016; Tallec et al., 2018). The influence of several media (ultrapure water, artificial or filtered  
98 natural seawater) and environmental factors (salinity and organic matter gradients) was explored  
99 with a temporal survey on their behavior in suspension for coping with environmental variability  
100 and to anticipate further ecotoxicology testing.

## 101 **2 Materials and Methods**

### 102 **2.1 Nanoplastics**

103 Three types of nanopolystyrene beads (50 nm) were used in this study: (i) without surface  
104 functionalization – PS-Plain; (ii) with carboxyl groups – PS-COOH; (iii) with amine groups – PS-NH<sub>2</sub>.  
105 All NP were purchased from Polysciences/Bangs Laboratories and stored at 4°C prior to  
106 experiments. Polymer types were previously confirmed by Raman microspectroscopy analysis  
107 (Tallec et al., 2018). Commercial suspensions were supplied in ultrapure water (UW) with a small  
108 amount of surfactant (<0.1%; Tween-20<sup>®</sup>). All tests were performed with the same batch of  
109 particles.

## 110 **2.2 Dynamic Light Scattering (DLS) analyses**

111 For DLS analyses, commercial suspensions of NP were diluted in UW at a stock concentration of  
112 1,000 mg.L<sup>-1</sup> then at a work concentration of 100 mg.L<sup>-1</sup> in the selected media according to Tallec et  
113 al. (2018). It was the optimal concentration allowing high reproducibility and sufficient detection  
114 level of particles by DLS. The need to use high particles concentration to reach a high measurement  
115 accuracy is common in the field of nanoparticles such as PS (50 mg.L<sup>-1</sup>; Cai et al., 2018); iron oxide  
116 (200 mg.L<sup>-1</sup>; Chekli et al., 2013); gold (20 mg.L<sup>-1</sup>; Liu et al., 2012); titanium oxide (50 – 80 mg.L<sup>-1</sup>;  
117 French et al., 2009; Loosli et al., 2013) and zinc oxide (100 mg.L<sup>-1</sup>; Mohd Omar et al., 2014). The  
118 analysis of colloidal fraction from environmental matrices required ultrafiltration (Minténig et al.,  
119 2018). The ultrafiltration factor used by Ter Halle et al. (2017) to detect traces of NP from the North  
120 Atlantic Gyre was 200. Therefore the concentration of our working solutions could be extrapolated  
121 inversely leading to an environmental value theoretically equivalent to 500 µg.L<sup>-1</sup>.

122 DLS measurements were performed with a nano-Zetasizer ZS (Malvern Instruments, UK) using an  
123 angle of 173° Backscatter, a temperature of 20°C and an equilibration of 120 sec (González-  
124 Fernández et al., 2018). We used the implemented data analysis software to measure the mean size

125 of particles/aggregates (Z-average; nm), the aggregation state (polydispersity index – PDI; Arbitrary  
126 Units (A.U.)) and the mean surface charge ( $\zeta$ -potential; mV) of NP. When the PDI exceeded 0.2,  
127 particles were deemed to be aggregated. The accuracy of all measures was verified with the report  
128 quality from the implemented software and a counting rate being always higher than 100 kcps. The  
129 nanoplastic suspensions were injected in disposable fold capillary cells (DTS 1060C, Malvern  
130 Instruments, UK) with syringes to obtain a final volume of 1 mL. All measurements were performed  
131 in triplicate (13 runs and 10 sec.measure<sup>-1</sup> for PDI and Z-average; 40 runs and 10 sec.measure<sup>-1</sup> for  $\zeta$ -  
132 potential) according to González-Fernández et al. (2018). No effect of the surfactant is expected  
133 owing to its residual concentration (< 0.0001%) in all samples (Douglas et al., 1985).

## 134 **2.3 Effects of environmental conditions**

### 135 **2.3.1 Influence of media**

136 All particles were tested in three different media: (i) ultrapure water (UW; pH  $6.6 \pm 0.2$  ); (ii)  
137 artificial seawater (ASW; pH  $8.1 \pm 0.1$ ; 30 practical salinity unit [PSU]; NaCl 450 mM, KCl 10 mM,  
138 CaCl<sub>2</sub> 9 mM, MgCl<sub>2</sub> 30 mM and MgSO<sub>4</sub> 16 mM); (iii) 2- $\mu$ m filtered natural seawater from the Bay of  
139 Brest sampled in January 2018 (FSW; pH  $8.2 \pm 0.1$ ; PSU 32).

### 140 **2.3.2 Influence of the salinity**

141 To investigate the influence of the salinity, UW and ASW were used to create five intermediate  
142 solutions according to Loucaide et al. (2008): 0% (0 PSU; 0 mM), 25% (7.5 PSU; 163.25 mM), 50%  
143 (15 PSU; 326.5 mM), 75% (22.5 PSU; 489.75 mM) and 100% of ASW (30 PSU; 653 mM). Solutions  
144 were made one-day prior experiment and kept in dark condition at 4°C until use.



### 145 **2.3.3 Influence of the organic matter**

146 We used humic acid as a proxy of the presence of dissolved organic matter (DOM) in freshwater and  
147 estuarine/coastal environments (Baalousha et al., 2008; Cai et al., 2018; Fabrega et al., 2009).  
148 Humic acid (OM; CAS 1415-93-6) was purchased from Sigma-Aldrich. A stock solution of 1 g.L<sup>-1</sup> was  
149 prepared in UW or ASW and stirred during 24h then filtered on 0.2 µm (aPES membrane) according  
150 to Yang et al. (2013). For testing the influence of the OM concentration, work solutions were  
151 adjusted at three concentrations (1, 10 and 30 mg.L<sup>-1</sup>) corresponding to realistic aquatic  
152 concentrations (Cai et al., 2018). Measurements were performed immediately after contact (T0).

153 NP behavior was also observed over time in UW and ASW alone and with organic matter (UW+OM  
154 and ASW+OM) at the intermediate concentration (10 mg.mL<sup>-1</sup>). Measurements were performed at  
155 T0, T24h and T48h.

### 156 **2.4 Statistical analyses**

157 Statistical analyses and graphical representations were conducted using the R Software (R Core  
158 Team, 2016). Before statistical comparisons, normality and homoscedasticity were screened with  
159 the Shapiro-Wilk and Levene's methods, respectively. All analyses were operated using one-way  
160 ANOVA followed by pairwise comparisons (Tukey's method) when needed. Effects of treatment on  
161 the size average were performed only when the PDI was greater than a threshold set to 0.2  
162 indicating the start of an aggregation. The significance threshold was set at a p-value < 0.05. Data  
163 are expressed as the mean ± standard deviation (SD).

## 164 **3 Results**

### 165 **3.1 Influence of media**

166 The PS-NH<sub>2</sub> stayed at a nanometric scale in all media:  $53.3 \pm 2.3$  nm in UW,  $52.5 \pm 0.5$  nm in ASW  
167 and  $67.9 \pm 0.8$  nm in FSW (Figure 1A). A small aggregation was observed in FSW (PDI > 0.2; ANOVA,  
168 F= 110.8, p-value < 0.001). In contrast, the aggregation level of the PS-COOH and PS-Plain solutions  
169 increased significantly (ANOVA; PS-COOH: F= 135.3, p-value < 0.001; PS-Plain: F= 358.5, p-value <  
170 0.001) following the same trend between each medium (Figure 1A). Particles stayed at a  
171 nanometric scale (PS-COOH;  $63.4 \pm 3.43$  nm; PS-Plain:  $56.0 \pm 0.2$  nm; PDI < 0.2) only in UW and  
172 formed microscale aggregates in ASW (PS-COOH:  $1,835.0 \pm 240.0$  nm; PS-Plain:  $2,106.7 \pm 75.4$  nm)  
173 and FSW (PS-COOH:  $4,530.3 \pm 528.0$  nm; PS-Plain:  $4,810.3 \pm 370.2$  nm).

174 The  $\zeta$ -potential of all particles was significantly different between UW and seawater (ANOVA; PS-  
175 NH<sub>2</sub>: F= 91.3, p-value < 0.001; PS-COOH: F =71.5; p-value < 0.001; PS-Plain: F= 51.7, p-value < 0.001),  
176 the values were buffered in seawater (Figure 1B). For the PS-NH<sub>2</sub>, in ASW and FSW, a mean  
177 reduction of 69% of the particle surface charge was observed in comparison with UW ( $58.0 \pm 2.5$   
178 mV). For the PS-COOH, the  $\zeta$ -potential increased by 20% and 70% in ASW and FSW, respectively, as  
179 compared to UW ( $-40.7 \pm 3.4$  mV). Similarly for the PS-Plain, a significant increase of 11% was  
180 observed in ASW and 34% in FSW compared to UW ( $-43.1 \pm 0.8$  mV).

### 181 **3.2 Influence of the salinity**

182 No statistical effect (ANOVA, p-value > 0.05) of the ionic strength gradient was observed on the  
183 aggregation of PS-NH<sub>2</sub> particles (mean value =  $51.2 \pm 1.8$  nm) (Figure 2A). Despite this observation,  
184 the increase of salinity caused a significant reduction (ANOVA, F= 35.7, p-value < 0.001) of the  $\zeta$ -  
185 potential with values corresponding to 58.0, 39.2, 32.8, 22.5 and 27.4 mV at 0, 163.25, 326.5,  
186 489.75 and 653 mM, respectively (Figure 2B).

187 A significant effect on aggregation level of the PS-COOH (ANOVA,  $F= 74.5$ ,  $p\text{-value} < 0.001$ ) and PS-  
188 Plain (ANOVA,  $F= 253.5$ ,  $p\text{-value} < 0.001$ ) suspensions was demonstrated from 489.75 mM and  
189 326.5 mM, respectively (Figure 2A). The size of the PS-COOH suspension increased 18-fold from  
190 0/163.25/326.5 mM (mean value =  $70.8 \pm 25.9$  nm) to 653 mM ( $1,835 \pm 240$  nm). Close results were  
191 observed for the PS-Plain with a 38-fold increase from 0/163.25 mM (mean value =  $53.8 \pm 6.4$  nm)  
192 to 653 mM ( $2,106 \pm 75$  nm). A significant effect in the  $\zeta$ -potential was observed with an increasing  
193 trend along the gradient for both PS-COOH (ANOVA,  $F= 6.7$ ;  $p\text{-value} < 0.001$ ) and PS-Plain (ANOVA,  
194  $F= 15$ ;  $p\text{-value} < 0.001$ ) from 326.5 mM (Figure 2B). Compared to 0 mM where PS-COOH and PS-  
195 Plain had a mean  $\zeta$ -potential of  $-43.9$  and  $-42.2$  mV, respectively, a maximal increase of 26% and  
196 28% was observed at the highest ionic strength (PS-COOH:  $-32.6 \pm 3.5$  mV; PS-Plain:  $-30.2 \pm 2.1$  mV).

### 197 **3.3 Influence of the organic matter**

198 The highest concentration of organic matter ( $30 \text{ mg.L}^{-1}$ ) in UW affected significantly (ANOVA,  $F=$   
199  $1426$ ,  $p\text{-value} < 0.001$ ;  $\text{PDI} > 0.2$ ) the average size of the PS-NH<sub>2</sub> solution leading to the formation of  
200 aggregates with a mean size of  $99.4 \pm 1.8$  nm while no aggregation was reported for lower levels of  
201 organic matter ( $1$  and  $10 \text{ mg.L}^{-1}$ ; mean value =  $56.1 \pm 1.4$  nm) (Figure 3). The addition of organic  
202 matter decreased significantly (ANOVA,  $F= 1497$ ,  $p\text{-value} < 0.001$ ) in a dose-response manner the  $\zeta$ -  
203 potential of the PS-NH<sub>2</sub> in UW ( $1 \text{ mg.L}^{-1}$ :  $46.2 \pm 0.7$  mV;  $10 \text{ mg.L}^{-1}$ :  $40.5 \pm 0.4$  mV;  $30 \text{ mg.L}^{-1}$ :  $24.3 \pm$   
204  $0.4$  mV) (Figure 4). In contrast, no effect (ANOVA,  $p\text{-value} > 0.05$ ) of the organic matter on the PS-  
205 NH<sub>2</sub> (average size and  $\zeta$ -potential) was observed in ASW (Figure 3 & 4).

206 For other NP (PS-COOH and PS-Plain), their size and surface charge were statistically similar  
207 (ANOVA,  $p\text{-value} > 0.05$ ) regardless of the organic matter contents. In UW, the PS-COOH and PS-  
208 Plain suspension remained at a nanoscale without aggregation with an average size and a  $\zeta$ -

209 potential of  $57.3 \pm 1.5$  nm/ $-42.5 \pm 5.0$  mV and  $51.8 \pm 1.3$  nm/ $-43.0 \pm 4.7$  mV, respectively (Figures 3  
210 & 4). In ASW and regardless of the OM concentrations, aggregation reached  $1,777 \pm 34$  nm for the  
211 PS-COOH and  $2,082 \pm 206$  nm for the PS-Plain with a  $\zeta$ -potential of  $-27.9 \pm 1.6$  mV and  $-35.7 \pm 3.8$   
212 mV, respectively.

### 213 3.4 Temporal stability

214 The PS-NH<sub>2</sub> remained at a nanometric scale in all media (UW and ASW with or without OM) but  
215 formed small homo-aggregates (PDI > 0.2) at T48h in UW ( $67.4 \pm 1.6$  nm; ANOVA, F= 33.1, p-value <  
216 0.01), ASW ( $71.8 \pm 1.3$  nm; ANOVA, F= 181.5, p-value < 0.001) and ASW+OM ( $96.6 \pm 1.6$  nm;  
217 ANOVA, F: 1300, p-value < 0.001) (Figure 5). In UW+OM at T48h, the PS-NH<sub>2</sub> did not aggregate and  
218 displayed a size of  $56.3 \pm 0.3$  nm (ANOVA, p-value > 0.05). Concerning the  $\zeta$ -potential, no effect was  
219 recorded in ASW with or without organic matter (mean value =  $23.8 \pm 2.9$  mV) (Figure 6). However,  
220 significant decreases in UW (-12%; ANOVA, F= 47.9, p-value < 0.001) and UW+OM (-13%; ANOVA,  
221 F= 148.2, p-value < 0.001) were observed at T48h (UW:  $45.7 \pm 1.3$  mV; UW+OM:  $40.4 \pm 0.4$  mV)  
222 compared to T0 (UW:  $51.4 \pm 0.4$  mV; UW+OM:  $46.7 \pm 0.8$  mV).

223 The PS-COOH and PS-Plain suspensions stayed at a nanoscale in UW (+/- OM) despite the apparition  
224 of small aggregates (PDI > 0.2) at 48h for the PS-COOH in UW ( $68.2 \pm 1.7$  nm; ANOVA, F= 50.1, p-  
225 value < 0.001) and UW+OM ( $77.4 \pm 4.6$  nm; ANOVA, F= 38.9, p-value < 0.001) and at 24h for the PS-  
226 Plain only in UW ( $63.3 \pm 5.0$  nm; ANOVA, F= 16.6, p-value < 0.01) (Figure 5). In both ASW (ANOVA,  
227 F= 42, p-value < 0.001) and ASW+OM (ANOVA, F= 50.6, p-value < 0.001), the PS-COOH formed  
228 aggregates with an average size close to 2,000 nm at 24h and 4,000 nm at 48h (Figure 5). The size of  
229 the PS-Plain's aggregates in ASW was  $1,884 \pm 223$  nm but exceeded the size limit of the zetasizer (10  
230  $\mu$ m) at T24h and T48h, thus no statistical analysis was performed even if a clear trend is obvious

231 with bigger aggregates at T24h and T48h compared to T0. In ASW+OM, the PS-Plain formed bigger  
232 aggregates (ANOVA,  $F= 209.7$ ,  $p\text{-value} < 0.001$ ) at T24h ( $8,343 \pm 228.3$  nm) compared to T0 ( $3,034 \pm$   
233  $187$  nm) and T48h ( $4,637 \pm 480$  nm). For PS-COOH, the  $\zeta$ -potential increased significantly in UW  
234 (+32%; ANOVA,  $F= 23.73$ ,  $p\text{-value} < 0.01$ ) and UW+OM (+27%; ANOVA,  $F= 18.04$ ,  $p\text{-value} < 0.01$ ) at  
235 T48h compared to T0 (Figure 6). A significant increase of the  $\zeta$ -potential was observed over the time  
236 for the PS-Plain in UW (+55%; ANOVA,  $F= 108.9$ ,  $p\text{-value} < 0.001$ ). In UW+OM, no change was  
237 observed at T0 ( $-24.3 \pm 0.4$  mV) and T48h ( $-29.9 \pm 0.4$  mV) but a significantly lower value (ANOVA,  
238  $F= 8.1$ ,  $p\text{-value} < 0.05$ ) was recorded at T24h ( $-45\%$ ;  $-48.8 \pm 1.3$  mV) (Figure 6). From 0 to 48h, no  
239 effect on the  $\zeta$ -potential (ANOVA,  $p\text{-value} > 0.05$ ) was observed in ASW with or without organic  
240 matter for the PS-COOH and PS-Plain (Figure 6).

#### 241 4 Discussion

242 In ultrapure water, all NP displayed a great stability and stayed at a nanometric scale over time  
243 because they presented a high positive or negative charge maintaining electrostatic repulsive forces  
244 limiting or reducing aggregation processes (El Badawy et al., 2010; Lin et al., 2010). In contrast,  
245 modifications of NP features (aggregation and  $\zeta$ -potential) were observed in the presence of salts.  
246 The behavior of the PS-COOH and PS-Plain suspensions was drastically affected in both artificial and  
247 natural seawater through the formation of microscale homo-aggregates. In contrast, the PS-NH<sub>2</sub>  
248 displayed high stability and stayed dispersed. Because particles were tested exactly in the same  
249 media, the observed behavior must be related to the surface functionalization as previously  
250 reported for other engineered nanomaterials (ENMs) (Liu et al., 2012). Consistent with our study,  
251 fast homo-aggregation of plain and carboxylate nano-polystyrene beads (25 and 50 nm) in seawater  
252 was previously demonstrated with aggregates larger than 1  $\mu\text{m}$  (Della Torre et al., 2014; Tallec et

253 al., 2018; Wegner et al., 2012). This large aggregation could have strong outcomes in aquatic  
254 environments because when the size of aggregates exceeds 1  $\mu\text{m}$ , nanoparticles loss their Brownian  
255 behavior in favor of sedimentation processes (Klaine et al., 2008). Results differed from recent  
256 studies using carboxylate and plain nanopolystyrene of 100 nm where no homo-aggregation was  
257 observed under an increasing ionic strength (Cai et al., 2018; González-Fernández et al., 2018). This  
258 difference can be explained by the specific features of the particles (*e.g.* size, surface chemistry and  
259 heterogeneity) (Alimi et al., 2018). Homo-aggregation in seawater is one of the most frequent  
260 behavior observed for nanomaterials (Christian et al., 2008). It is due to interactions between the  
261 negative surface charge of NP and cationic elements naturally present in seawater such as calcium  
262 or sodium ions (El Badawy et al., 2010). Hence, the  $\zeta$ -potential of the PS-COOH and PS-Plain became  
263 less negative in seawater decreasing the NP stability (Cai et al., 2018; Lin et al., 2010). Indeed, under  
264 the presence of salts, attractive forces (including van der Waals forces) and particles sticking  
265 efficiency increased according to the Derjaguin-Landau-Verwey-Overbeek (DLVO) model resulting in  
266 homo-aggregation (Alimi et al., 2018; Liu et al., 2012). Thus, the difference of salinity between  
267 artificial (30 PSU) and natural (32 PSU) seawater can explained the observed variation of  
268 aggregation level of the PS-COOH and PS-Plain. The ionic strength increased from ASW to FSW, as  
269 well as the screening of the repulsive electrostatic interactions. In the presence of an excess of  
270 added salt, the particles diffuse ion double layer reduces and possible specific ions condensation  
271 might occur and decrease the particles net surface charge density. Moreover, in natural seawater,  
272 other molecules such as extracellular polymeric substances (EPS) produced by bacteria can also  
273 intensify aggregation in comparison to an artificial and controlled medium (Summers et al., 2018).  
274 Regarding the PS-NH<sub>2</sub>, its strong stability in all media was presumably due to a positive coating  
275 characterized by a low effect of the ionic strength (particles and homo-aggregates stayed at a

276 nanometric scale) in comparison to the two other particles tested. Despite a decrease in the  $\zeta$ -  
277 potential when particles are suspended in seawater, it appears sufficiently high to ensure repulsive  
278 mechanism. Overall, the nature of the surface groups and the particles interface has a stronger  
279 influence than the nature of the core polymer on the particles aggregation (Liu et al., 2012). To  
280 thoroughly test this hypothesis, particles of different nature (PE, PP, PS, non-plastic material)  
281 presenting the same coating should be compared in controlled experimental solutions.

282 Humic substances (HA) – used as proxy of dissolved organic matter (DOM) – had negligible effect on  
283 the behavior of all particles in comparison to the presence of salts. Usually, organic matter tends to  
284 stabilize nanoparticles by increasing steric surface repulsive forces. The presence of cations can  
285 overcome and disrupt this stabilization by several mechanisms (*e.g.* bridging, electrical repulsion  
286 compression) promoting aggregation (Zhang et al., 2009). In agreement with our study, divalent  
287 cations ( $Mg^{2+}$  and  $Ca^{2+}$ ) triggered homo-aggregation of carbon nanotubes despite the presence of  
288 organic matter (reviewed by Christian et al., 2008). Only for the PS-Plain, HA had a stabilizing impact  
289 in seawater causing a partial disaggregation over the temporal experiment (between T24h and  
290 T48h) presumably due to an increase in the steric repulsion level. In contrast to previous studies  
291 using nanomaterials (Baalousha, 2009; Loosli et al., 2013), no shift in the  $\zeta$ -potential of NP was  
292 observed, explaining the negligible effect of the DOM. This fact is possibly linked to the  
293 concentration of HA used in the present study. At a concentration of  $100\text{ mg}\cdot\text{L}^{-1}$ , HA caused a shift in  
294 the  $\zeta$ -potential of iron oxide nanoparticles whilst no effect was perceived at  $10\text{ mg}\cdot\text{L}^{-1}$  (Baalousha,  
295 2009) which is consistent with our results at the same concentration. In ultrapure water, an  
296 adsorption of HA on the PS-NH<sub>2</sub> is hypothesized with a decrease of the  $\zeta$ -potential along the  
297 increasing gradient of HA doses. Indeed, the anionic groups of HA interact easily with the positive  
298 surface charge of the PS-NH<sub>2</sub>. This adsorption reduced the net charge density and resulted in a small

299 aggregation at the highest HA dose, even though the PS-NH<sub>2</sub> mean size distribution always stayed  
300 below 100 nm. Overall, DOM have limited effects on NP behavior in our experimental design but a  
301 recent study demonstrated that 25 nm NP can in reverse affect the assembling of DOM with  
302 suspected consequences on carbon flux in oceans according to the environmental concentration of  
303 NP (Chen et al., 2018).

304 The temporal analysis demonstrated that NP formed evolving dynamic structure. Indeed, an  
305 increase of the homo-aggregation level is reported over time for all NP tested in ASW and  
306 ASW+OM. Hence, it is highly plausible that NP will not be find individually in oceans. Owing to the  
307 high aggregation level observed here, if NP have a negative surface charge (acquired naturally or  
308 after weathering in rivers) as observed in marine environment (Fotopoulou and Karapanagioti,  
309 2012), the risk of an intake in oceans from freshwater appears therefore low. However, certain  
310 events can influence the retention of plastic particles in rivers such as flooding leading to a washout  
311 and a huge export of plastic debris to marine systems as recently described in rivers around urban  
312 area of the United Kingdom (70% of microplastics were exported by the flooding) (Hurley et al.,  
313 2018). Every year, between 1.15 and 2.41 MT of plastic debris go into oceans from rivers (Lebreton  
314 et al., 2017). Thus, in future models or experiments, it will be crucial to consider the behavior of NP  
315 with a temporal and seasonal aspect as reported with silver nanoparticles (Ellis et al., 2018).

316 In the context of increasing number of studies using NP to highlight their toxicity on freshwater  
317 (Besseling et al., 2014; Cui et al., 2017; Mattsson et al., 2017) and marine (Canesi et al., 2016; Tallec  
318 et al., 2018) organisms, this study revealed that it is unavoidable to properly characterize NP  
319 behavior in the experimental systems. Indeed, the risk assessment is completely modified if  
320 particles stayed individual or formed aggregates in the medium (Lowry et al., 2012). The presence of



321 aggregates for PS-COOH and PS-Plain in seawater would be part of their lower toxicity observed in  
322 Tallec et al. (2018) compared to PS-NH<sub>2</sub> for which we showed in the present study strong stability at  
323 nanometric scale. Then, because the PS-Plain formed microscale aggregates in seawater but not in  
324 freshwater, the low toxicity of the PS-Plain observed in our previous study on oyster planktonic-  
325 stages (Tallec et al., 2018) is far from comparable to the severe outcomes in the freshwater  
326 crustacean *Daphnia galeata* (Cui et al., 2017). Likewise, for a given biological model, various results  
327 may be observed according to the medium tested. For instance, the toxicity of the PS-NH<sub>2</sub> appeared  
328 stronger on the survival of the rotifer *Brachionus plicatilis* in artificial seawater ( $EC_{50} = 2.75 \pm 0.67$   
329  $\mu\text{g}\cdot\text{mL}^{-1}$ ) than in natural seawater ( $EC_{50} = 6.62 \pm 0.87 \mu\text{g}\cdot\text{mL}^{-1}$ ) (Manfra et al., 2017). Colloids, organic  
330 matter, macromolecules (e.g. EPS, proteins) or compounds released by organisms in the water, at  
331 their epithelium interface or inside the organism, are likely to interact with nanoparticles (corona  
332 formation) and change their behavior and bio-availability and consequently potential harmful  
333 effects (Lowry et al., 2012). Because, this corona can be specific according to the surface  
334 functionalization (Lundqvist et al., 2008), an examination of the influence of natural compounds  
335 may be one priority for the ecotoxicological community. For instance, changes in the toxicity of  
336 various nanomaterials such as polystyrene (Nasser & Lynch, 2016), titanium dioxide (Yang et al.,  
337 2012), silver (Fabrega et al., 2009), multiwalled carbon nanotubes (Edgington et al., 2010) were  
338 reported under co-exposures with humic acids or macromolecules.

339 We conclude that the surface functionalization is deemed to be a major parameter determining the  
340 fate of 50 nm NP in our experimental design and potentially in aquatic environments. The data  
341 presented here, complemented by previous studies including the recent publication of Cai et al.  
342 (2018) with 100 nm PS-Plain, emphasizes the need for a thorough characterization of NP  
343 considering at least size and coating, in relevant environments in order to better design

344 experiments, understand end-points and define toxicity thresholds for this new threat. More  
345 experiments are required to understand effect of other environmental conditions on NP behavior  
346 especially the weathering of particles which can affect their properties as previously observed with  
347 MP (Rummel et al., 2017). These results provide new experimental data to consider in the  
348 assessment of NP fate in future modeling studies.

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## 354 **6 Authors Contributions**

355 KT, AH, IPP, GB, CG-F designed experiments. KT, OB conducted experiments. DLS data were  
356 analyzed by KT and OB. Data were interpreted by OB, KT, AH, IPP, PS and GB. KT wrote the initial  
357 draft in concertation with AH and IPP. All authors read and contributed to the final manuscript.

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## 556 8 Figure Legend

557 **Figure 1.** Size average (nm; A) and  $\zeta$ -potential (mV; B) of three 50 nm nanoplastics (PS-NH<sub>2</sub>; PS-  
558 COOH; PS-Plain) in three media: ultrapure water (UW; white), artificial seawater (ASW; light grey)  
559 and 2- $\mu$ m filtered natural seawater (FSW; dark grey). DLS analysis were replicated 3 times and data

560 are given as mean  $\pm$  SD. Multiple pairwise comparisons were performed using the Tukey's HSD  
561 method; homogeneous groups share the same letter.

562 **Figure 2.** Average size (nm; A) and  $\zeta$ -potential (mV; B) of three 50 nm nanoplastics (PS-NH<sub>2</sub>; PS-  
563 COOH; PS-Plain) along a salinity gradient (0, 163.25, 326.5, 489.75, 653 mM). DLS analysis were  
564 replicated 3 times and data are given as mean  $\pm$  SD. Multiple pairwise comparisons were performed  
565 using the Tukey's HSD method; homogeneous groups share the same letter.

566 **Figure 3.** Average size (nm) of three 50 nm nanoplastics (PS-NH<sub>2</sub>; PS-COOH; PS-Plain) in two media  
567 (ultrapure water – UW or artificial seawater – ASW) with three different doses of organic matter: 1  
568 (white), 10 (light grey) and 30 (black) mg.L<sup>-1</sup>. DLS analysis were replicated 3 times and data are given  
569 as mean  $\pm$  SD. Multiple pairwise comparisons were performed using the Tukey's HSD method;  
570 homogeneous groups share the same letter.

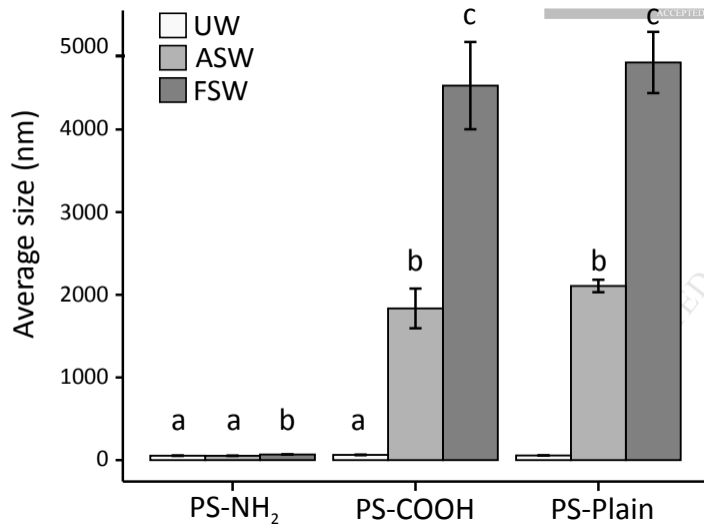
571 **Figure 4.**  $\zeta$ -potential (mV) of three 50 nm nanoplastics (PS-NH<sub>2</sub>; PS-COOH; PS-Plain) in two media  
572 (ultrapure water – UW or artificial seawater – ASW) according to three different doses of organic  
573 matter: 1 (white), 10 (light grey) and 30 (black) mg.L<sup>-1</sup>. DLS analysis were replicated 3 times and data  
574 are given as mean  $\pm$  SD. Multiple pairwise comparisons were performed using the Tukey's HSD  
575 method; homogeneous groups share the same letter.

576 **Figure 5.** Average size (nm) of three 50 nm nanoplastics (PS-NH<sub>2</sub>; PS-COOH; PS-Plain) over time (T0,  
577 T24h and T48h) in two media (ultrapure water – UW and artificial seawater – ASW) with or without  
578 organic matter (OM, 10 mg.L<sup>-1</sup>). DLS analysis were replicated 3 times and data are given as mean  $\pm$   
579 SD. Multiple pairwise comparisons were performed using the Tukey's HSD method; homogeneous  
580 groups share the same letter.

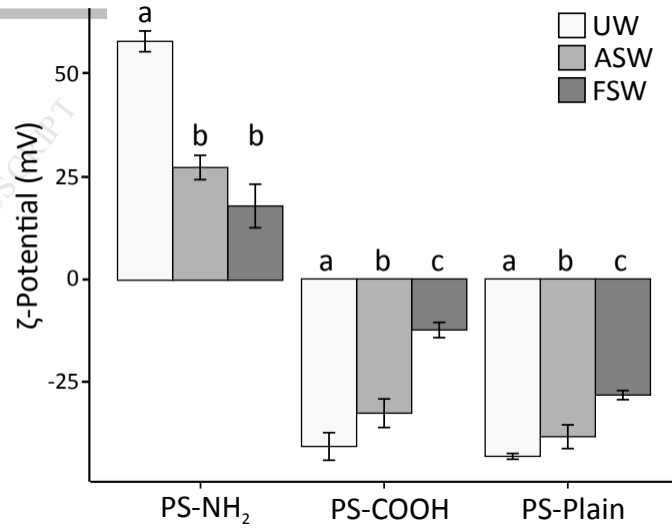
581 **Figure 6.**  $\zeta$ -potential (mV) of three 50 nm nanoplastics (PS-NH<sub>2</sub>; PS-COOH; PS-Plain) over time (T0,  
582 T24h and T48h) in two media (ultrapure water – UW and artificial seawater – ASW) with or without  
583 organic matter (OM, 10 mg.L<sup>-1</sup>). DLS analysis were replicated 3 times and data are given as mean  $\pm$   
584 SD. Multiple pairwise comparisons were performed using the Tukey's HSD method; homogeneous  
585 groups share the same letter.

586

A

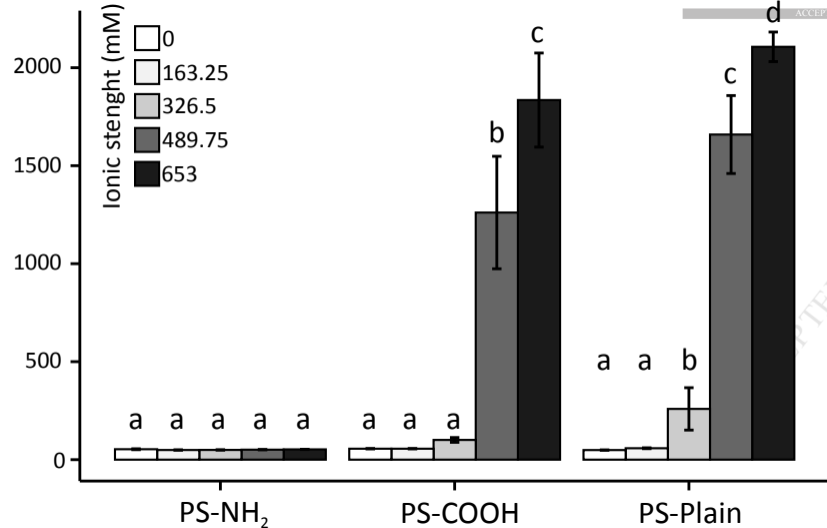
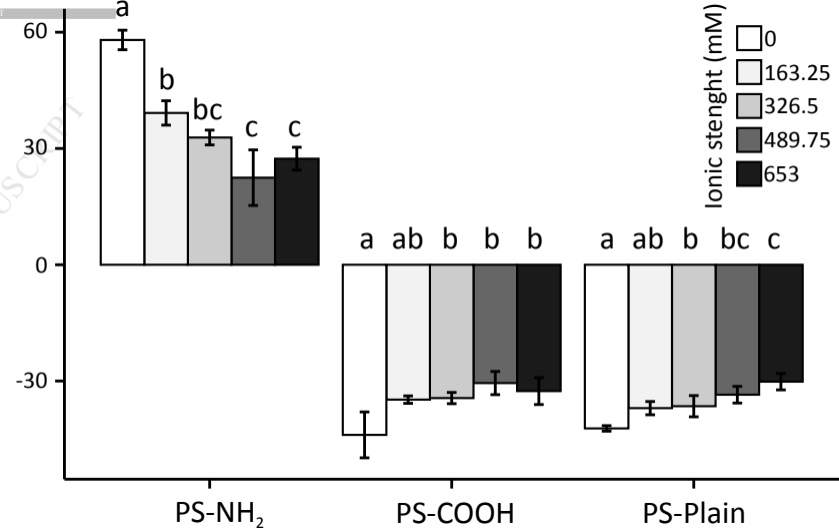


B



**A**

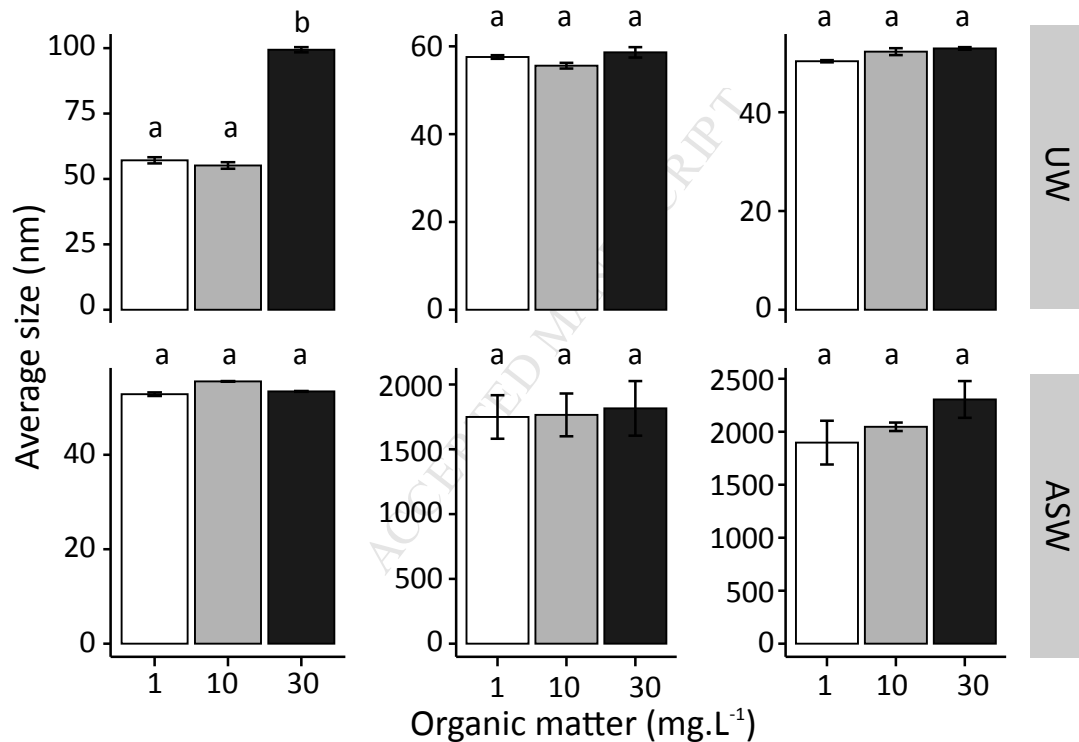
Average size (nm)

**B** $\zeta$ -Potential (mV)

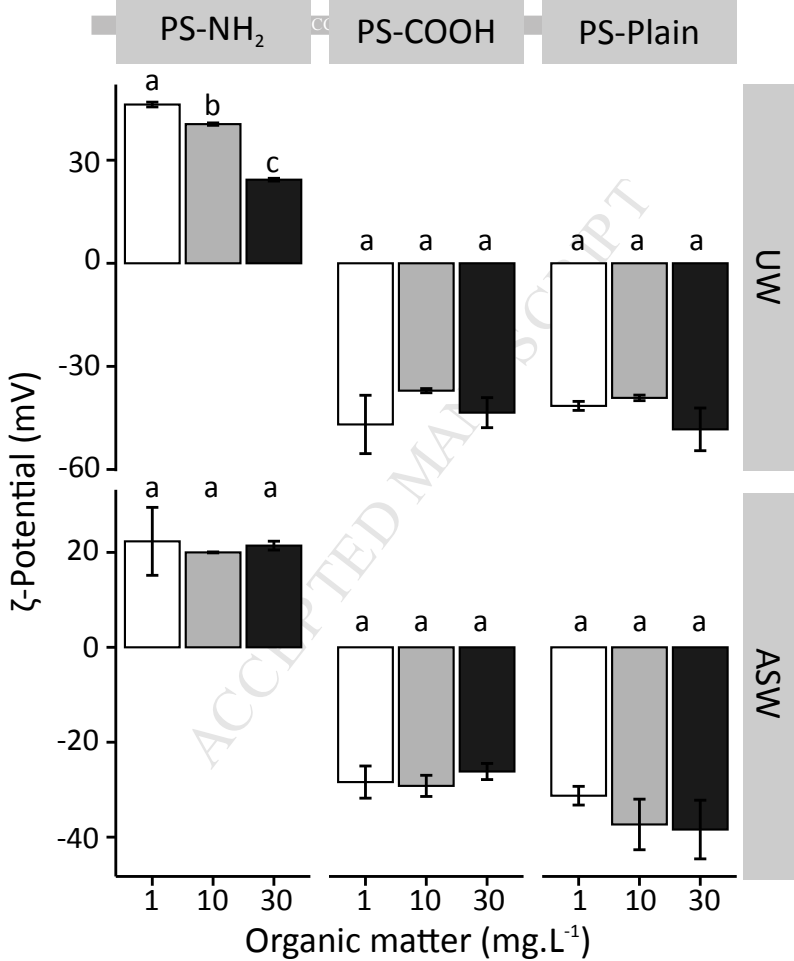
PS-NH<sub>2</sub>

PS-COOH

PS-Plain







PS-NH<sub>2</sub>

A

PS-COOH

T

PS-Plain

