# Comparative ecotoxicity of polystyrene nanoparticles in natural seawater and reconstituted seawater using the rotifer Brachionus plicatilis

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Abstract: The impact of nanoplastics using model polystyrene nanoparticles (PS NPs), anionic (PS-COOH) and cationic (PS-NH2), has been investigated on the marine rotifer Brachionus plicatilis, a major component of marine zooplanktonic species. The role of different surface charges in affecting PS NP behavior and toxicity has been considered in high ionic strength media. To this aim, the selected media were standardized reconstituted seawater (RSW) and natural sea water (NSW), the latter resembling more natural exposure scenarios. Hatched rotifer larvae were exposed for 24h and 48h to both PS NPs in the range of 0.5-50 ug/mL using PS NP suspensions made in RSW and NSW. No effects on lethality upon exposure to anionic NPs were observed despite a clear gut retention was evident in all exposed rotifers. On the contrary, cationic NPs caused lethality to rotifer larvae but LC50 values resulted lower in rotifers exposed in RSW (LC50=2.75±0.67 µg/ml) compared to those exposed in NSW (LC50=6.62±0.87 µg/ml). PS NPs showed similar pattern of aggregation in both high ionic strenght media (RSW and NSW) but while anionic NPs resulted in large microscale aggregates (Z-average 1109 ± 128 nm and 998±67 nm respectively), cationic NP aggregates were still in nano-size forms (93.99 ± 11.22 nm and 108.3 ± 12.79 nm). Both PDI and Z-potential of PS NPs slightly differed in the two media suggesting a role of their different surface charges in affecting their behavior and stability. Our findings confirm the role of surface charges in nanoplastic behaviour in salt water media and provide a first evidence of a different toxicity in rotifers using artificial media (RSW) compared to natural one (NSW). Such evidence poses the question on how to select the best medium in standardized ecotoxicity assays in order to properly assess their hazard to marine life in natural environmental scenarios.

Dear Editor,

please receive the ms titled "Comparative ecotoxicity of polystyrene nanoparticles in natural seawater and reconstituted seawater using the rotifer *Brachionus plicatilis*" authored by L. Manfra, A. Rotini, E. Bergami, G. Grassi, C. Faleri, I. Corsi, which has been revised according to the reviewers' suggestions.

The final version of the ms has been approved by all Authors.

Sincerely,

Loredana Manfra on behalf of all the authors.

**Reviewers' comments:**

**Reviewer #1:**

This is a very interesting and timely study. The article is very descriptive and needs to be re-written in order to focus the discussion for each result. Some examples:

"Differences in surface charge seem to be a first key factor in determining both behaviour and ecotoxicity of PS NPs, in agreement with literature studies." Seems to be? In agreement with literature studies? Witch studies? Please re-write the sentence or remove it.

We rewritten this sentence according to the reviewer criticism as follows: "Differences in surface charge seem to be a key factor in determining both behaviour and ecotoxicity of PS NPs according to our previous findings in which we identify significant differences in PS nanoparticles toxicity based on their different surface charge (Bergami et al., 2016; Della Torre et al., 2014; Lundqvist et al 2008)" (Lines 286-289).

A detailed explanation of why such hypothesis has been confirmed by the results of the present study has been provided also in the discussion where how surface charge can drive PS NPs toxicity is well reported (Lines 328-354), also supported by human studies (Liu et al 2011; Lundqvist et al., 2008) (Lines 322-327).

Several studies have evaluated the toxicity of NPs to aquatic organisms, some of them used marine species but the most reported no specific information on exposure media. Seawater was not often classified as natural or reconstituted/artificial and the behavior of NPs, or PS NPs, was rarely characterized in the exposure media. Witch studies? Please re-write or remove the sentences.

We rewritten this sentence according to the reviewer criticism as follows: "Several studies have evaluated the toxicity of PS NPs to aquatic organisms, some of them used marine species but often seawater was not classified as natural or reconstituted/artificial (Ward and Kach 2009; Wegner et al., 2012) and the behaviour of PS NPs was not characterized in the exposure media (Ward and Kach 2009; Snell and Hicks, 2011; Cole and Galloway, 2015)" (Lines 380-383).

**Reviewer #2:**

The Manuscript (EES-17-849) by Manfra et al. Titled: "Comparative ecotoxicity of polystyrene nanoparticles in natural seawater and reconstituted seawater using the rotifer Brachionus plicatilis"

General comments: This is a paper where authors describe the behaviours of two selected nano-plastic, the model polystyrene nanoparticles (PS NPs), anionic (PS-COOH) and cationic (PS-NH2) in natural sea water and reconstituted sea water aiming to provide new insights into the standardization of reconstituted sea waters in environmental exposure tests. Moreover, the toxicological effect of the two nano plastics was investigated in the marine rotifer Brachionus plicatilis after 24 h and 48 h of exposure. The investigation of the effects of plastic surface charge on its behaviour in RSW as well as in NSW and the evaluation of their toxicity on marine organisms is extremely interesting and welcome by all researchers involved in this field.

However, there are two main points that must be addressed and clarified:

* No effects on lethality upon exposure to anionic NPs were observed despite a clear gut retention, while cationic NPs caused lethality! Indeed Surface charge could be very determinant in term of NP behavior in waters but in term of toxicity to living organisms, how could this be explained!?

*Did the mortality observed in Brachionus individuals is due to a physical effect of there is particular hypothetical explanation of the biological mode of action (in view of its cationic/anionic specificities)? The discussion section should be better targeted toward the explanation of the two reported points!

The discussion regarding how the surface charge of PS NPs can be involved in the toxicity mechanisms as well as the possible biological modes of action has been included. Please refer to the new text as follows: "These evidences suggest that a positive charge is definitely a critical parameter in cellular toxicity (Bexiga et al., 2013). The rotifer mortality observed in the present study represents a further confirmation of the role of positive surface charges of PS NPs in causing toxicity, irrespective of the exposure medium. According to described above literature, the surface charge is determinant in NP aggregation, which in turn affects bioavailability and thus toxicity to living organisms. The size-dependent toxicity of PS micro- and nanoparticles has been demonstrated in vivo and in vitro toxicity tests with rotifers (Jeong et al., 2016). In this study, PS-COOH formed micro-scale aggregates in the media and the strong aggregation pattern could be related to reduced bioavailability of PS-COOH and explain its lack of toxicity. In fact, PS-COOH were found accumulated and excreted in marine organisms as brine shrimps, rotifers, sea urchins causing mainly sub-lethal

effects (i.e. behavioral, physiological and bio- chemical) and suggesting a potential trophic transfer along marine trophic webs. In contrast, PS-NH2 particles were still present as NP aggregates in the media with increased abilities to penetrate in tissues/cells and longer retention times. Thus, their smaller size plays an important role in determining the toxicity, affecting seriously the development and growth of these species.
The positive surface charge of PS NPs is known to be involved in the mechanisms of toxicity. PS-NH2 can bind with high affinity to lipid bilayers on the cell membrane in favour of cellular uptake via endocytosis causing toxicity (Van Lehn and Alexander-Katz, 2011; Lin and Alexander-Katz, 2013; Wang et al., 2013). Regarding the possible biological modes of action, in our previous studies was hypothesized a direct toxicity of PS-NH2 caused by up-regulation of genes involved in brine shrimp larval molting and energy metabolism (i.e. clap and cstb, Bergami et al., 2017). Furthermore, Bergami et al. (2017), observed the decrease in algal growth rate suggesting that photosynthesis might be impaired and ROS production triggered in D. tertiolecta species. Della Torre et al (2014) demonstrated that PS-NH2 were able to elicit developmental and growth defects in sea urchin through the induction of target genes related to stress (i.e. hsp70) and apoptosis (cas8). In addition, in the rotifer B. koreanus antioxidant-related enzymes and MAPK signaling pathways were activated in response to PS NP exposure (Jeong et al., 2016).” (Lines 328-354)

Some minor comments regarding the way the Introduction section is organized:
*in my opinion it should be reduced

Introduction has been reduced from 1144 to 937 words.

*the last paragraph of the introduction section should be presented before the aim of the work.

The paragraph has been moved accordingly.

## Highlights

PS NP behavior in seawaters and ecotoxicity on marine zooplankton

Surface charge and exposure medium as critical factors

Suitability of natural seawater for resembling more natural exposure scenarios

How to select the best medium in standardized ecotoxicity assays

# Comparative ecotoxicity of polystyrene nanoparticles in natural seawater and reconstituted seawater using the rotifer *Brachionus plicatilis*

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**Abstract**

The impact of nanoplastics using model polystyrene nanoparticles (PS NPs), anionic (PS-COOH) and cationic (PS-$NH_2$), has been investigated on the marine rotifer *Brachionus plicatilis*, a major component of marine zooplanktonic species. The role of different surface charges in affecting PS NP behavior and toxicity has been considered in high ionic strength media. To this aim, the selected media were standardized reconstituted seawater (RSW) and natural sea water (NSW), the latter resembling more natural exposure scenarios. Hatched rotifer larvae were exposed for 24h and 48h to both PS NPs in the range of 0.5-50 ug/mL using PS NP suspensions made in RSW and NSW. No effects on lethality upon exposure to anionic NPs were observed despite a clear gut retention was evident in all exposed rotifers. On the contrary, cationic NPs caused lethality to rotifer larvae but $LC_{50}$ values resulted lower in rotifers exposed in RSW ($LC_{50}$=2.75±0.67 µg/ml) compared to those exposed in NSW ($LC_{50}$=6.62±0.87 µg/ml). PS NPs showed similar pattern of aggregation in both high ionic strenght media (RSW and NSW) but while anionic NPs resulted in large microscale aggregates (Z-average 1109 ± 128 nm and 998±67 nm respectively), cationic NP aggregates were still in nano-size forms (93.99 ± 11.22 nm and 108.3 ± 12.79 nm). Both PDI and Z-potential of PS NPs slightly differed in the two media suggesting a role of their different surface charges in affecting their behavior and stability. Our findings confirm the role of surface charges in nanoplastic behaviour in salt water media and provide a first evidence of a different toxicity in rotifers using artificial media (RSW) compared to natural one (NSW). Such evidence poses the question on how to select the best medium in standardized ecotoxicity assays in order to properly assess their hazard to marine life in natural environmental scenarios.

**Key-words**

nanoplastics; polystyrene; rotifer; ecotoxicity; PS NP surface charge, suitable testing medium

## 1. Introduction

Plastic represents the prevalent marine litter (Barnes et al., 2009), with an estimated release of 8 millions tons per year and around 300,000 tons expected to be in open-ocean surface waters including fragments of smaller sizes from microscopic to nanoscopic (Jambeck JR et al. 2015; Mattsson et al., 2015). Nanoplastic, referred as plastic particles in the <100 nm size range, is probably the less known fraction of marine litter but potentially the most hazardous to marine life due to nanodimensional peculiar properties which make them largely different from the same polymer type in bulk form (Koelmans et al., 2015). Among plastic polymers found in marine litter, polystyrene (PS) has been reported to be the most abundant plastic type (OSPAR, 2015) and account for 6-7.8% of total plastic production worldwide with an annual production of over 23 million tons per year (Lithner et al., 2011). As far as for most of the plastic polymers ending up into the environment as wastes, PS items are subjected to both abiotic and biotic weathering processes which lead to their degradation and fragmentation in smaller fragments of micrometric (microplastics, <5 mm) and nanometric size (nanofragmentation, nanoparticles <100 nm) (Shim et al., 2014). Therefore, PS nanofragments/particles will inevitably constitute a significant portion of floating plastic debris in ocean's surface waters. Polystyrene nanoparticles (PS NPs) are also directly discharged as wastes into the oceans being used for various applications such as biosensors, in photonics and self-assembling structures and even in consumer products and developed in research for medical applications as nanospheres and nanocapsules for drug delivery (Salvati et al., 2011; Loos et al., 2014).

Based on their nano-design (size, morphology and surface charges), PS NPs own various functional properties which allow them to be uptaken and internalized by cells, but more important to affect cell functioning leading to severe cell damage as apoptosis (Bramini et al., 2014; Wang et al., 2013; Bexiga et al., 2011). In the last years a number of studies performed under controlled laboratory conditions demonstrated mild to severe impacts in marine organisms from planktonic species

(microalgae, microcrustaceans and sea urchin embryos) to filter-feeders (bivalves) and bottom grazers. PS NPs mainly affect several biological and ecological targets as microalgal growth, early development of sea urchin embryos, accumulation, mortality and multiple molting in brine shrimp, cell functional parameters and immune responses in bivalves (Ward and Kach, 2009; Wegner et al., 2012; Della Torre et al., 2014; Canesi et al., 2015; Cole and Galloway, 2015; Bergami et al., 2016; Sjollema et al., 2016). Size-dependent toxicity was reported in a study on rotifers in which larger particles (≥100 nm) induced gut accumulation until excretion, while smaller (50 nm) were uptaken by cells, transferred from mothers to the extruded eggs with consequences as reduction in reproduction and feeding rate (Snell and Hicks, 2011). Similarly, rotifers exposed to micro- and nano PS particles showed significant size dependent adverse effects on growth, reproduction, reactive oxygen species (ROS) production and antioxidant response (Jeong et al., 2016). These recent evidences underline the need to understand pathways for animal and human exposure, as well as assess their potential trophic transfer along marine food chain.

Nanoscale properties of PS significantly affect their behaviour in aquatic media as for instance in seawater and therefore their bioavailability, uptake, ultimate accumulation and toxicity. Indeed, their ecotoxicity may rely on their peculiar features such as particle size, polymer type, surface charge but also on the properties of the receiving water medium which affects their behaviour (e.g. aggregation). Based on the current criticism expressed towards the suitability of ecotoxicology standardized bioassays for engineered NPs and nanomaterials (NMs) hazard testing, it is mandatory to adopt new approaches as including the use of natural exposure media as test condition in order to more accurately predict any risk associated to their release into the natural environment (Bour et al., 2015).

Reconstituted seawater (RSW) is the most used exposure medium in standardized methods for evaluating ecotoxicity of conventional contaminants. It is considered a good quality water, easily available in the laboratory, suitable to a wide range of test organisms, and its use allows to obtain repeatable and reproducible data (ASTM 2013). RSW reduces the variability of the exposure media

during development, intercalibration and standardization of methods. Despite being suitable for molecules, transformation occurring in natural seawater (NSW), which represents the real environmental scenario in which PS NPs may end up, is not be taken into account. According to recent findings in both freshwater and salt water media, natural water components such as colloids and NOM, under a variety of physico-chemical conditions including pH and ionic strength, will significantly affect NP behaviour and consequently toxicity (Nasser et al., 2016; Canesi et al., 2016). Therefore, the behaviour of PS NPs in NSW may be different than in RSW and, thus, the relevance of ecotoxicological studies performed with artificial media need to be fully addressed and discussed.

Rotifers are suitable model organisms being high sensitive to a large number of environmental contaminants, they populate both freshwater and coastal marine ecosystems and have a high ecological relevance being a food source for fish larvae and other marine predators including marine birds. PS NPs impact on rotifers will have serious repercussion not only on the species itself but therefore on the marine food chain.

The aim of the present study is to investigate the ecotoxicity of two types of PS NPs, anionic (PS-COOH) and cationic (PS-$NH_2$), in RSW and NSW media using the marine rotifer *Brachionus plicatilis* as model species. Acute toxicity test with rotifers has been chosen for its robustness among standardized methods used in ecotoxicology and as reliable bioassays (ASTM 2004; ISO 2016).

## 2. Materials and methods

### 2.1 PS NP behavior in RSW and NSW exposure media

40 nm green fluorescently labeled carboxylated polystyrene nanoparticles (PS-COOH NPs, 40 nm size) (505 nm excitation, 515 nm emission) were purchased from Invitrogen. 50 nm unlabeled amino modified polystyrene nanoparticles (PS-$NH_2$ NPs) were purchased from Bangs Laboratories Inc. Anionic (PS-COOH) and cationic (PS-$NH_2$) PS NPs have been widely used as recommended

polymeric material in both nanotoxicology and ecotoxicological studies (Stone et al., 2010; Bhattacharya et al., 2010, Besseling et al., 2014, Della Torre et al., 2014, Cole and Galloway, 2015, Canesi et al., 2015, Canesi et al., 2016, Pinsino et al., 2017).

Primary characterization of PS NPs was performed as reported in Bergami et al. (2016). Secondary characterization of PS NPs in RSW and NSW media and in comparison with milliQ was performed using Dynamic Light Scattering (DLS, Malvern instruments), combined with the Zetasizer Nano Series software, version 7.02 (Particular Sciences, UK). Z-average (nm), Polydispersity Index (PDI, dimensionless) and Zeta (ζ-) potential (mV) were measured as key parameters describing NP behaviour in complex environmental media (SCENIHR, 2007; Stone et al., 2010). Measurements were carried out in triplicate, each containing 11 runs of 10 second for size parameters, 20 runs for ζ-potential.

RSW was prepared following the ASTM guide (2004) for conducting acute ecotoxicological tests with rotifers: 26.01g NaCl, 0.83g KCl, 1.24g $CaCl_2$, 4.53g $MgCl_2 \cdot 6H_2O$, 5.50g $MgSO_4 \cdot 7H_2O$, and 0.39g $NaHCO_3$ were added to 1L to high-quality deionized water, filtered at 0.22 µm and stored at 4°C until used. NSW was collected from a marine uncontaminated site located inside the Tuscan Archipelago, NW Tyrrhenian Sea, stored in the dark and filtered at 0.45 µm before use. Physico-chemical parameters of NSW were recorded before running ecotoxicological tests as follows: salinity 38‰, total organic carbon 1.3%, water hardness 1940 mg/L, oxygen 6.6 mg/L. PS particle sizes and morphology were addressed by means of transmission electron microscopy (TEM) through a Tecnai G2 Spirit operating at 100 KV. PS-COOH and PS-$NH_2$ were dispersed in milliQ, RSW and NSW at a concentration of 10 µg/ml. After 48h incubation, a 10 µL-drop of NP suspensions was placed on a formovar/carbon-coated copper grids and dried before imaging. NPs suspended in RSW and NSW were extensively washed before deposition and drying, while milliQ dispersions were straightforwardly imaged. Given the high ionic strength nature of both RSW and NSW, a washing procedure was required to reduce the overall content of salts, which otherwise would have crystallized during sample drying yielding images of poor quality. Briefly, after

incubation in NSW, PS NPs were centrifuged gently for 20 min and suspended in milliQ, this procedure was done for three times. Finally, the newly achieved suspensions were dried and analysed by TEM.

### *2.2 Ecotoxicity tests*

Certified dehydrated cysts of *B. plicatilis* were purchased from MicroBioTests (Ghent, Belgium) (48h $LC_{50}$ of 213 µg/ml [181-245] for $K_2Cr_2O_7$ reference toxicant). Cysts were incubated in RSW (15‰) at 25°C and 3000 lux illumination for 24-26h. Before the test (2h), hatched larvae were transferred in fresh RSW (34‰) and NSW (38‰) to adapt the rotifers at the different salinities used in the experiment. According to a previous study performed on the microcrustacean brine shrimp *Artemia franciscana* by Bergami and co-authors (2016), a range of PS NPs was tested: 0.5-1-5-10-25-50 µg/ml. Our goal in selecting PS NP concentrations above those expected to be present in the ocean's surface waters was to provide insights into mechanism of toxicity as well as pathways of exposure which could be compared to those observed in other aquatic models as well as human cell lines (Nasset et al., 2016; Bergami et al., 2016; Besseling et al., 2014, Della Torre et al., 2014, Cole and Galloway, 2015, Canesi et al., 2015, Canesi et al., 2016, Pinsino et al., 2017; Bexiga et al., 2011; Wang et al., 2013). For each concentration, three replicates were set and two independent experiments were run for each PS NPs. PS NP final suspensions were prepared in both media as NSW and RSW, from stock solutions of PS-COOH (50 mg/ml) and PS-$NH_2$ (100 mg/ml) in milliQ and quickly vortexed after sonication (10 min, 60 watt, 47 kHz; Branson Ultrasonic Baths).

PS NP final suspensions in RSW and NSW were prepared from the stock solutions and quickly vortexed without sonication prior to use based on our previous findings which showed any significant change in PS NP size in NSW with sonication (see Della Torre et al., 2014 SI). Moreover, such dispersion will resemble more realistic natural scenarios for nanoplastic dispersion in ocean's surface waters.

Bioassays were performed using 24-well plates. Ten rotifers per replicate were exposed to 1 ml of each PS NP suspension in NSW and RSW. Plates were incubated at 25±1°C in darkness.

After 24h and 48h, the number of alive and dead rotifers was counted under stereomicroscope, at 10× to 15× magnification. Lack of movement, including mastax and foot movement, was considered as death. The tests showing 10% mortality or less in the control were considered valid.

A recovery test was performed only for fluorescently labelled PS-COOH in NSW based on previous findings on brine shrimp larvae, which showed a clear accumulation inside gut upon PS-COOH exposure. After 48h of exposure the rotifers were transferred in clean NSW (without PS-COOH) and rotifers were then processed as described above for counting. Wells were then fixed by adding 10 µl of a lugol:ethanol solution at 25±1 °C in the dark according to Snell and Hicks (2011). Rotifers were observed using an Axioskop 40 Carl Zeiss microscope at 200× magnification with a 10 Zeiss filter (excitation: 450-490 nm; emission: 515-565 nm) and digital images were taken using a Canon Power Shot camera at 10x.

### *2.3 $LC_{50}$ and data analysis*

The lethal concentrations ($LC_{50}$) were estimated by the Spearman-Karber method. One-way analysis of variance (ANOVA), followed by post-hoc t-test were performed using stats package in R software (2015) to test treatment effect and significant pairwise differences among treatments and control.

## 3. Results and discussion

Our study aims at evaluating the ecotoxicity of cationic (PS-COOH) and anionic (PS-$NH_2$) PS NPs in two exposure media, the artificial and standardized reconstituted sea water (RSW) and the natural sea water (NSW) using the marine rotifer *B. plicatilis* as model organism.

### *3.1 Characterization of PS NPs*

The characterization of PS NPs through DLS analysis showed an optimal dispersion of both PS NPs in MilliQ, with Z-Average values of 58±2 nm for PS-COOH and 54±1 nm for PS-$NH_2$ and PDI < 0.192. Moreover, the ζ-potential for both PS NPs (around 50 mV in absolute value) clearly indicates a good stability of PS NPs in milliQ.

Anionic NPs form microscale aggregates in both RSW and NSW, as indicated by the Z-Average values of 998±67 nm in NSW and slightly higher in RSW (1109±128 nm).

The low ζ-potential absolute values (around -10 mV) also confirmed the aggregation state and instability of the particle.

On the contrary, positive PS-$NH_2$ were still present as NPs in both media used in the ecotoxicity tests, despite higher PDI and lower ζ-potential values compared to MilliQ. In particular, a difference in Z-Average values of 108±13 nm in NSW and 94±11 nm in RSW was found. Z-Average is the most stable parameter produced by DLS analysis, showing the hydrodynamic diameter of the particles in suspension. Concerning nano-sized PS-$NH_2$, the slight increase in Z-Average observed in the natural medium compared to the reconstituted one may be an indication of the stronger interactions of the single NP with other compounds naturally present in the medium and absent in RSW. Further details of results obtained from DLS are reported in Table 1.

The intensity-based size distributions from data obtained by DLS confirmed this peculiar aggregation pattern of both PS NPs in NSW and RSW with microscale aggregates (900-1000 nm) for PS-COOH opposed to nanoscale aggregates (90-100 nm) of PS-$NH_2$ (Fig. 1). These results are in agreement with our previous findings showing the different behaviour of PS-$NH_2$ and PS-COOH in NSW (Della Torre et al., 2014; Bergami et al., 2016) and confirm a similar pattern of each PS NP in RSW. The high ionic strength of seawater (i.e. 38‰ for NSW and 34‰ RSW respectively) is probably driving such aggregation state compared to milliQ.

TEM images, shown in Figure 2, clearly support the DLS data in which both PS-COOH and PS-$NH_2$ NPs resulted well dispersed in milliQ medium (a and d) and less dispersed and highly aggregated in RSW and NSW for PS-COOH and far less for PS-$NH_2$ (b, c and e, f). A slight but not

significant evidence of a thin translucent coating at the border of aggregates and around single NPs resembling a corona-like structure (*eco-coronas*) for PS NPs suspended in NSW compared to RSW could be seen. Therefore, considering the similar high ionic strength in the two media, the presence of NOM in NSW might be responsible for such effects. Further studies are thus required to better clarify the NOM involvement in affecting such peculiar effects in PS NPs dispersed in NSW media.

**Table 1.** Polystyrene nanoparticle behaviour in milli-Q water (milliQ), Natural Sea Water (NSW) and reconstituted sea water (RSW) dispersions by DLS. Data are referred to PS NPs concentration of 50 µg/ml and values reported as average ± standard deviation of 3 independent measurements.

**Figure 1.** Intensity-based size distributions on logarithmic scale from data obtained by DLS analysis of 40 nm PS-COOH (a) and 50 nm PS-$NH_2$ (b) in milli-Q water (milliQ), Natural Sea Water (NSW) and reconstituted sea water (RSW). Z-average (nm) at 50 µg/ml. For each medium, one independent measurement is shown. The graphs were edited using GraphPad Prism5.

**Figure 2.** TEM images of 10 µg/ml PS-COOH (a,b,c) and PS-$NH_2$ (d,e,f) NPs dispersed in milliQ, RSW and NSW respectively. Scale bar: 100 nm.

### *3.2 Ecotoxicity of PS NPs*

The role of functionalizations (negative/positive charge) and exposure media (artificial/natural sea water) on the toxicity was assessed.

The toxicity of PS-COOH and PS-$NH_2$ NPs has been evaluated by observing the mortality rate of *B. plicatilis* after 24-48h of exposure in NSW and RSW media.

All results were acceptable as the mortality was ≤10% in all control groups according to standardized protocols (ASTM 2004; ISO 2016).

Different effects on rotifers were observed upon exposure to anionic and cationic PS NPs and also regardless to the their suspension in the artificial and natural sea water media.

We observed different aggregation and ecotoxicity of PS NPs in function of their surface charge: microscale aggregates and no mortality for anionic PS-COOH vs nanoscale aggregates and mortality for cationic PS-$NH_2$.

In particular, anionic PS NPs did not cause mortality to rotifer larvae in the range of tested concentrations (0–50 µg/ml), therefore $LC_{50}$ was assessed as >50 µg/ml but not calculated. PS-COOH gut retention was the most relevant impact upon exposure in both media. In fact, fluorescent aggregates were detected inside the body of exposed rotifers after 48h (Fig. 3b) and still present even after the recovery period (Figure 3d), suggesting that the time used for testing an excretion was too short and that PS-COOH can be retained in the gut of the larvae for long time. Future studies are therefore recommended in order to address clearance rate and mechanisms, since these findings showed a clear bioaccumulation of nanoplastics in the body of rotifers and might support a potential biomagnification along the marine food webs being rotifers a food source for fish larvae and other marine predators including marine birds.

Contrarily no sign of accumulation/clearance of PS-$NH_2$ was observed in this study since tests were performed using no fluorescent PS-$NH_2$ for a better comparison with previous studies done in marine species and human cell lines.

**Figure 3**. Observation of *B. plicatilis* rotifers in the ecotoxicity test (a. control and b. 5 µg/ml PS-COOH) and in the recovery test (c. control and d. 5 µg/ml PS-COOH). Scale bar 50 µm.

However, cationic PS NPs caused mortality at concentrations ≥2.5 µg/ml. While no differences were observed after 24h in rotifers exposed to PS-$NH_2$ suspensions in both media, at 48h lower $LC_{50}$s were found in organisms exposed to PS-$NH_2$ in RSW compared to those in NSW. The $LC_{50}$ values of three independent tests were reproducible (CV <25%); the mean 24h $LC_{50}$s were in fact

13.17±0.71 µg/ml for PS-$NH_2$ suspended in RSW and 13.04±0.60 µg/ml (NSW) while the mean 48h $LC_{50}$s were 2.75±0.67 µg/ml for PS-$NH_2$ suspended in RSW and 6.62±0.87µg/ml for those in NSW. Despite such differences, a concentration-dependent increase in mortality was observed in both media (ANOVA: $F=111.8$, $p<0.001$, for NSW; $F=105.9$, $p<0.001$, for RSW) (Figure 4). In rotifers exposed to RSW, the mortality was significant compared to controls already at 5 µg/ml (t test: $t=9.0122$, $p<0.01$) while for NSW significant differences were observed only from 10 µg/ml (t test: $t=6.9921$, $p<0.02$).

**Figure 4**. Mortality rate (%) of *B. plicatilis* rotifers after 24h (a) and 48h (b) of exposure in Natural Sea Water (NSW) and reconstituted sea water (RSW). First concentrations showing significant differences with control based on pairwise, post-hoc t test are indicated with asterisks, for both exposure media.

Differences in surface charge seem to be a key factor in determining both behaviour and ecotoxicity of PS NPs according to our previous findings in which we identify significant differences in PS nanoparticles toxicity based on their different surface charge (Bergami et al., 2016, 2017; Della Torre et al., 2014; Lundqvist et al 2008).

In fact, no lethal and sub-lethal effects have been reported upon exposures to anionic PS NP up to 100 µg/ml but their gut retention. Recent studies conducted in rotifers exposed to uncharged PS NPs demonstrated size-dependent effects. Joeng et al (2016) observed that antioxidant-related enzymes and MAPK signaling pathways were activated in response to PS NP exposures, in a size-dependent manner, causing adverse effects on rotifer growth, reproduction and lifespan. Snell and Hicks (2011) found larger PS NPs accumulated in the rotifer stomach and intestine (100 nm) and smaller (50 nm) able to enter tissues and pass from mother to the extruded eggs, and affect reproduction and feeding rate. Body retention of PS NPs has been already reported for other marine species, with no associated severe effect but sub-lethal responses. Ward and Kach (2009) reported that aggregates

present in seawater significantly enhance the possibility of ingestion of 100 nm PS NPs by suspension-feeding bivalves (mussels, *Mytilus edulis*; oysters, *Crassostrea virginica*). Wegner et al. (2012) reported a concentration-dependent effect of 30 nm PS NPs on feeding behaviour of *Mytilus edulis* in seawater due to progressively reduced filtering activity and higher pseudofeaces production. In our previous study (Della Torre et al., 2014), we observed a significant retention of PS-COOH in sea urchin embryos without no detrimental effect up to 50 µg/ml. As well, no mortality of brine shrimp larvae exposed to PS-COOH was observed but their sequestration inside the gut lumen after 48h and 14d of exposure (Bergami et al., 2016, 2017). Nevertheless, considering that PS NPs form large aggregates of µm size, their retention and complete or partial elimination may reduce the normal uptake capacity and lead to physiological impairments (Bergami et al., 2016). Therefore, it is worth to deepen the knowledge on processes of accumulation and excretion since they could be crucial for a better understanding of PS NP fate in marine trophic webs. Since the ingestion of PS NPs may cause their transfer from the water column to organisms and sediments, this might lead to trophic transfer and biomagnifications of nanoplastics in long-term exposure scenarios.

On the opposite, cationic PS NP exposures caused severe damages in other marine invertebrate species as for instance on development of sea urchin embryos (Della Torre et al., 2014), on molting and swimming of brine shrimp larvae (Bergami et al., 2016, 2017), on algal growth (Bergami et al., 2017) and in mussel's hemocytes as clear signs which anticipate cell death (Canesi et al, 2016). In particular our $LC_{50}$s calculated for rotifer larvae exposed to PS-$NH_2$ suspended in RSW are similar or even lower than those reported to cause embryotoxicity in sea urchins (24h $EC_{50}$ = 3.82 µg/ml; 48h $EC_{50}$= 2.61 µg/ml) (Della Torre et al., 2014). Except for the work done in mussels, all previous studies were performed by comparing toxicity of the two type of PS NPs and in general PS-$NH_2$ resulted always to be more toxic than PS-COOH in all marine species. Such findings were in agreement with previous studies performed in human cell lines, where toxicity was mainly referred to the differences in surface charges of PS NPs (Liu et al., 2011) and surface properties played a

very significant role in determining the nanoparticle coronas on the different particles of identical materials (Lundqvist et al., 2008). PS-$NH_2$ have been shown to cause disruption of cell membrane, generate oxidative stress and induce cell death in human cells (Frolich et al., 2012).

These evidences suggest that a positive charge is definitely a critical parameter in cellular toxicity (Bexiga et al., 2013). The rotifer mortality observed in the present study represents a further confirmation of the role of positive surface charges of PS NPs in causing toxicity, irrespective of the exposure medium. According to described above literature, the surface charge is determinant in NP aggregation, which in turn affects bioavailability and thus toxicity to living organisms. The size-dependent toxicity of PS micro- and nanoparticles has been demonstrated *in vivo* and *in vitro* toxicity tests with rotifers (Jeong et al., 2016). In this study, PS-COOH formed micro-scale aggregates in the media and the strong aggregation pattern could be related to reduced bioavailability of PS-COOH and explain its lack of toxicity. In fact, PS-COOH were found accumulated and excreted in marine organisms as brine shrimps, rotifers, sea urchins causing mainly sub-lethal effects (i.e. behavioral, physiological and bio- chemical) and suggesting a potential trophic transfer along marine trophic webs. In contrast, PS-$NH_2$ particles were still present as NP aggregates in the media with increased abilities to penetrate in tissues/cells and longer retention times. Thus, their smaller size plays an important role in determining the toxicity, affecting seriously the development and growth of these species.

The positive surface charge of PS NPs is known to be involved in the mechanisms of toxicity. PS-$NH_2$ can bind with high affinity to lipid bilayers on the cell membrane in favour of cellular uptake via endocytosis  causing toxicity (Van Lehn and Alexander-Katz, 2011; Lin and Alexander-Katz, 2013; Wang et al., 2013). Regarding the possible biological modes of action, in our previous studies was hypothesized a direct toxicity of PS-$NH_2$ caused by up-regulation of genes involved in brine shrimp larval molting and energy metabolism (i.e. *clap* and *cstb*, Bergami et al., 2017). Furthermore, Bergami et al. (2017), observed the decrease in algal growth rate suggesting that photosynthesis might be impaired and ROS production triggered in *D. tertiolecta* species. Della

Torre et al (2014) demonstrated that PS-$NH_2$ were able to elicit developmental and growth defects in sea urchin through the induction of target genes related to stress (i.e. hsp70) and apoptosis (cas8). In addition, in the rotifer *B. koreanus* antioxidant-related enzymes and MAPK signaling pathways were activated in response to PS NP exposure (Jeong et al., 2016).

Behaviour and toxicity of NPs, including PS, are known to be modulated by their intrinsic properties and exposure medium composition (Petersen et al., 2015). None of literature studies neither characterize the behavior of the PS NPs in the media nor indicate a functionalization as for instance the presence of surface charges. Our previous studies have been directed to assess the role of functionalization but in this paper we studied it in relation with different ionic strength media. Exactly, in the European FP7 project MARINA, modifications of exposure media (i.e. no shaking or organic matter addition) have been proposed in order to obtain more realistic environmentally conditions during ecotoxicological tests (Hund-Rinke et al., 2016; Holden et al., 2016).

Despite PS-$NH_2$ showed a lower toxicity in NSW to rotifers, no important differences in their dispersion were observed between NSW and RSW (see Figure 1,2), despite a slight increase in their mean hydrodynamic diameter in the natural medium. Therefore, the observed difference in PS-$NH_2$ toxicity can relies upon the close interactions between the positive surface charges and molecules present in NSW, which may have an influence in PS toxicity. As far as the observed differences in toxicity of PS-$NH_2$ in the two media, RSW being higher than in NSW, the potential role of biomolecules in affecting NPs interaction with cells and therefore toxicity should be addressed due to their presence in NSW. Indeed, these interactions may modify behaviour of PS-$NH_2$ as natural polymeric substances and other biomolecules in seawater gave a new character to the PS-$NH_2$ (Canesi & Corsi, 2016).

According to what recently described in freshwater media, the protein *corona* has a strong influenced in NP uptake by filter-feeding organisms (Nasser et al., 2016). No information so far are currently available on the interactions of functionalized PS NPs with NSW and the formation of an *eco-corona* upon contact with specific NSW components as colloids and NOM, which are absent in

RSW. PS-$NH_2$ links with these NSW components, driven mainly by electrostatic forces between the positively charged NPs and negatively charged organic matter might significantly affect bioavailability and thus ecotoxicity, as observed in rotifer.

Several studies have evaluated the toxicity of PS NPs to aquatic organisms, some of them used marine species but often seawater was not classified as natural or reconstituted/artificial (Ward and Kach 2009; Wegner et al., 2012) and the behaviour of PS NPs was not characterized in the exposure media (Ward and Kach 2009; Snell and Hicks, 2011; Cole and Galloway, 2015). Park et al. (2014) compared behaviour and ecotoxicity of gold NPs in standard test media and natural waters using freshwater species. These authors observed that the aggregation of NPs was influenced by environmental factors such as pH, organic matter and ions dissolved in NSW. They stated the need for toxicity tests on engineered nanoparticles to use standard media more realistic and representative of natural system. To the best of our knowledge, this is the first study that investigates and compares the behaviour and the ecotoxicity of differently charged PS NPs in two exposure media (NSW vs RSW) by using a marine organism. The presence/absence of an *eco-corona*-like structures potentially affecting the toxicity in NSW and RSW will be object to further studies.

## 4. **Conclusions**

The different surface charge of PS NPs may lead to different impacts on marine biota in terms of accumulation and toxicity, which deserve more attention. Positively charged PS-$NH_2$ showed a nano-aggregation state and high mortality in rotifers, while negatively charged PS-COOH showed micro-aggregates and accumulation inside organisms with no acute toxicity. This biodisposition of nanoplastics in marine organisms might lead to adverse effects not only on the single specie but also on the whole marine food web.

Our findings also underline that the choice of exposure medium can be a critical factor in ecotoxicological tests focused on the impact of NMs/NPs such as nanoplastics.

PS NPs resulted less toxic to rotifers in NSW exposure media compared to RSW thus stressing the need to further discuss how to best conduct ecotoxicity tests by using environmental realistic scenarios as for instance with natural sea water in which the presence of colloids, organic matter and proteins makes more realistic behaviour and perhaps affecting bioavailability and toxicity.

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