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Nano-sized polystyrene affects feeding, behavior and physiology of brine shrimp *Artemia franciscana* larvae

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**Abstract**

Nano-sized polymers as polystyrene (PS) constitute one of the main challenges for marine ecosystems, since they can distribute along the whole water column affecting planktonic species and consequently disrupting the energy flow of marine ecosystems. Nowadays very little knowledge is available on the impact of nano-sized plastics on marine organisms. Therefore, the present study aims to evaluate the effects of 40 nm anionic carboxylated (PS-COOH) and 50 nm cationic amino (PS-NH2) polystyrene nanoparticles (PS NPs) on brine shrimp *Artemia franciscana* larvae. No signs of mortality were observed at 48 h of exposure for both PS NPs at nauplius stage but several sub-lethal effects were evident. PS-COOH (5–100 μg/ml) resulted massively sequestered inside the gut lumen of larvae (48 h) probably limiting food intake. Some of them were lately excreted as fecal pellets but not a full release was observed. Likewise, PS-NH2 (5–100 μg/ml) accumulated in larvae (48 h) but also adsorbed at the surface of sensory antennules and appendages probably hampering larval motility. In addition, larvae exposed to PS-NH2 undergo multiple molting events during 48 h of exposure compared to controls. The activation of a defense mechanism based on a physiological process able to release toxic cationic NPs (PS-NH2) from the body can be hypothesized. The general observed accumulation of PS NPs within the gut during the 48 h of exposure indicates a continuous bioavailability of nano-sized PS for planktonic species as well as a potential transfer along the trophic web. Therefore, nano-sized PS might be able to impair food uptake (feeding), behavior (motility) and physiology (multiple molting) of brine shrimp larvae with consequences not only at organism and population level but on the overall ecosystem based on the key role of zooplankton on marine food webs.

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**1. Introduction**

Several studies estimate that trillions of plastics are floating all over the oceans (Eriksen et al., 2014), representing one of the most important threats for marine ecosystems (Cozar et al., 2014). Micro (<5 mm) and nanoplastics (<100 nm) resulting also from weathering and fragmentation processes of macro-debris have been acknowledged as the most dangerous for marine wildlife since they might be easily ingested causing chemical and physical effects to marine organisms (Cole et al., 2013). Smaller plastic pieces can be uptaken and retained by small invertebrates, leading to bioaccumulation (Browne et al., 2011; Lee et al., 2013; Ward and Kach, 2009; Wright et al., 2013a), toxicity (Cole et al., 2014), but also trophic transfer to top-predators with potential impact for marine ecosystems as a whole (Farrell and Nelson, 2013; Setala et al., 2014; Watts et al., 2014). Concerning the Mediterranean basin, one thousand tons of plastic debris have been recently reported with a frequency comparable to the five subtropical ocean gyres (Cozar et al., 2015). Therefore, it is necessary to gain a deeper insight into the impact of small plastics including nano-sized, since EU member states must develop activities to reach “good environmental status, GES” by 2020, as main goals of the Marine Strategy Framework Directive (Directive 2008/56/EC) (Galgani et al., 2010). Evidences of harmful effects of plastics are mostly restricted to observation on individual specimens and larger debris (micro and macroplastics) but concerns have been raised about physico-chemical effects of nanoplastics for single species up to ecosystem-wide impacts (UNEP, 2011). Both micro and nanoplastics are expected to increase consistently with time in the sea and oceans worldwide and important questions regarding sources, fate
and biological effects need to be answered. While microplastics are quite well studied, the occurrence as well as effects of nanosized particles are almost unknown and are raising concern due to expected increasing abundance in sea water (Cozar et al., 2014) and their intrinsic properties (Matranga and Corsi, 2012; Wright et al., 2013b). Polystyrene (PS) is one of the most largely used plastics worldwide, with an annual production of over 23 million tons per year (considering PS, high-impact PS and expanded PS) (Lithner et al., 2011). This polymer persists for several hundred years in the environment and undergoes to extremely slow depolymerization in marine waters (Andrady, 2003; Innocenti, 2003), thus leading to the formation of micro and nano-debris (Bandypadhyay and Basak, 2007; Hofer, 2008). Therefore, PS might pose a serious hazard to marine organisms due to the properties of the styrene monomer known as carcinogenic and endocrine disruptor (Lithner et al., 2011). These findings identified PS debris as potential multiple stressor in marine habitats, especially when available for ingestion by marine wildlife. Recent studies showed also higher sorption of Polycyclic Aromatic Hydrocarbons (PAHs) and Polychlorinated Biphenyls (PCBs) to nanosized PS compared to other plastics found in the marine environment (Rochman et al., 2013; Velzeboer et al., 2014).

In the last decades, PS based nanomaterials have been largely synthesized for several applications including packaging and nanomedicine (Bramini et al., 2014; Salvati et al., 2011; Silvestre et al., 2011). PS NPs refer to particles at nanoscale dimension with a PS core and variable functional groups, which determine their chemical reactivity and particle surface charge (Nowack and Bucheli, 2007). Common functionalized PS NPs include anionic carboxylated (–COOH) and cationic unsaturated amino (–NH₂) (Casado et al., 2013; Loos et al., 2014). Several studies revealed their cellular uptake in vitro models with human cell lines (Bramini et al., 2014; Lesniak et al., 2010; Liu et al., 2011; Lunov et al., 2011; Rossi et al., 2014), but also cytotoxicity and apoptosis in particular for PS-NH₂ (Bexiga et al., 2011; Wang et al., 2013a). Based on the current data, PS NPs uptake and toxicity depend on their intrinsic properties such as size and surface charges which affect their interaction with exposure media (Della Torre et al., 2014a).

Marine invertebrates are among the primary biological targets of nanoplastics, being exposed both to polymeric beads in suspension, as planktonic larvae, and to the fraction in sediments, as adult organisms (Manzo et al., 2013; Matranga and Corsi, 2012; Moore, 2006). Microplastics ingestion have been recently documented in several marine species including zooplankton therefore it is expected that they would be more severely exposed to nanosized floating debris (Chua et al., 2014; Cole et al., 2013; Lee et al., 2013; Murray and Cowie, 2011). Nanoscale materials (1–100 nm range) may end up to a significant aggregation in sea water due to counterbalance of several parameters as pH, salts, natural organic matter (NOM) and colloids with size and surface chemistry (i.e. charges) of the nanoparticle itself. Such aggregates may sink along sea column and reach marine sediment despite but they still undergo vertical repartition with consequent buoyancy due to strong water currents (i.e. upwelling) or by ingestion by planktonic organisms (Corsi et al., 2014). Based on recent evidences of suspended nanoscale materials in sea water, bottom grazers and filter-feeders species are expected to be exposed to high concentrations of plastic debris in their natural environment (Wright et al., 2013b). Our recent findings on selected PS NPs showed that both accumulation and toxicity affect the early life stages of development of the Mediterranean sea urchin Paracentrotus lividus depending on NPs surface charges and their aggregation in sea water (Della Torre et al., 2014a).

Specie-specific sensitivities including toxicity between 55 and 110 nm polyethyleneimine-PS NPs for aquatic organisms has been recently reported by Casado et al., 2013. Likewise, 70 nm PS-COOH NPs significantly affect algal growth and reproductive success of Daphnia magna through diet (Besseling et al., 2014). The few contributions on marine species show a significant accumulation in the gut of rotifers (Snell and Hicks, 2011) and bivalves (Ward and Kach, 2009; Wegner et al., 2012) as well as in sea urchin embryos, which were the first evidence of toxicity reported for the PS-NH₂ (Della Torre et al., 2014a). Therefore, beside the potential in nanotechnology, PS NPs represent an important source of primary nanoplastics entering in marine environment and based on the observed toxicity more studies should investigate their impact on organisms belonging to different trophic levels (Handy et al., 2008; Klaine et al., 2008; Moore, 2006). Although commonly applied as model positively charged NPs in nanosafety studies, it is important to stress that PS-NH₂ are a special surface functionalised variant of standard PS and the presence of similar positively charged NPs in plastic degradation products has not been fully determined yet.

Within marine model species, microcrustaceans are highly recommended in ecotoxicological studies being numerous and planktonic so expected to be exposed to nano-sized floating debris including the low-density PS NPs. Any negative impact on such key trophic level might disrupt the energy flow in marine ecosystems.

Brine shrimp Artemia franciscana, a filter-feeding anostracan microcrustacean is typical of inland salt water bodies but also temperate coastal areas and largely used in ecotoxicology studies for acute toxicity testing as model marine zooplankton species (EPA, 2002; Nunes et al., 2006; Persoone and Wells, 1987).

Up to date, the few recent studies conducted using this species show some variability in the observed effects towards NPs exposure (e.g. CeO₂, carbon black, graphene, Ag and other metal oxides) likely due to differences in the NPs chemical nature, size, surface properties as well as aggregation in the exposure water media (Arulvasu et al., 2014; Ates et al., 2013a, 2013b; Auffan et al., 2013; Gambardella et al., 2014; Petti et al., 2014; Rodd et al., 2014). Brine shrimps are non-selective filter feeders that can efficiently graze over a wide range of particles sizes, thus likely including synthetic nanomaterials and nanoplastics (Makridis and Vadstein, 1999).

The aim of the present study is to evaluate the uptake and distribution of 40 nm anionic carboxylated (PS-COOH) and 50 nm cationic amino modified (PS-NH₂) PS NPs on brine shrimp A. franciscana using larvae mortality test (Nunes et al., 2006).

2. Materials and methods

2.1. Physico-chemical characterization of PS-COOH and PS-NH₂ NPs

40 nm green carboxylated polystyrene nanoparticles (PS-COOH) (505 nm excitation, 515 nm emission) were purchased from Invitrogen. Unlabeled and blue fluorescently labeled (385 nm excitation, 410 nm emission) 50 nm amino modified polystyrene (PS-NH₂) NPs were purchased from Bangs Laboratories and Sigma, respectively. Fluorescently labeled PS beads have been recommended as priority test material to be developed and used for ecotoxicological studies (Stone et al., 2010) and investigated within the FP7 Research Infrastructure QualityNano (www.qualitynano.eu) (Wang et al., 2013a, 2013b). Fluorescent PS micro and nano-beads have been also widely used to investigate the impact of micro and nanoplastics on marine biota (Cole et al., 2013; Lee et al., 2013; Della Torre et al., 2014a). Nevertheless their utilization has not always been combined with in-depth secondary characterization in the natural media and the role of their functionalized groups and thus surface charge have been rarely taken into account. This could lead to an incomplete comprehension of
toxicity results. Although negative surface charged nanoplastics as PS-COOH have been suggested as the most widespread in the environment (Besseling et al., 2014), no data are currently on their fate and toxicity to marine biota.

In this study, PS-\(\text{NH}_2\) were also considered as positively charged nanoplastics, although they are a special surface functionali-\(\text{}\)sed variant of common PS and the presence of similar positively charged NPs as plastic degradation products has not been determined.

TEM was applied for primary particle diameter identification of PS NPs (Philips Morgagni 268D electronics, at 80 kV and equipped with a MegaView II Ccd camera). Dynamic Light Scattering (Malvern instruments) was used for size (\(Z\)-average and polydispersity index, PDI) and zeta (\(\zeta\)-) potential (mV) (Zetasizer Nano Series software, version 7.02, Particular Sciences, UK). Measurements have been performed in triplicate, each containing 11 runs of 10 s for determining \(Z\)-average, 20 runs for the \(\zeta\)-potential.

Natural sea water (NSW) was collected from a pristine area in the Tuscan archipelago and used for PS NPs suspension preparation and without PS NPs as a control. Physico-chemical parameters including some aquatic contaminants of NSW samples used in the study have been reported in Table 3. PS NPs suspensions were prepared in 0.45 \(\mu\)m filtered NSW (\(T = 25 \pm 1^\circ\text{C}, \text{s}alinity 38\%\), \(pH\) 8.3, conductivity 6 S/m) and quickly vortexed prior to use but not sonicated.

PS NPs concentrations for toxicity tests were chosen based on those used in previous studies on in vitro cell models (Bexiga et al., 2011; Salvat et al., 2011; Wang et al., 2013a). Despite data on environmental concentrations of similar particles in sea water are not available, these concentrations may be far above real exposure conditions. PS-\(\text{NH}_2\) caused apoptosis in 1321N1 human cells at 50 \(\mu\)g/ml (Bexiga et al., 2014), but in our previous study (Della Torre et al., 2014a) we observed induction of an apoptotic pathway in sea urchin developing embryos already at 3 \(\mu\)g/ml, thus raising concern regarding the impact of lower PS NPs concentrations in marine organisms.

2.2. Ecotoxicity tests

Acute toxicity test using \(A.\ franciscana\) larvae has been developed as standard methods (CNR, 2003; EPA, 2002; Vanhaecke and Persone, 1981) for assessing the lethality of contaminants at the first stages of development (up to Instar III nauplius), since 48 h old specimens is considered the most sensitive larval end-point (Barahona and Sánchez-Fortún, 1996). Recovery experiments were also performed by transfer PS NPs exposed brine shrimp larvae after 48 h in clean NSW and left there for 24 h. For both acute toxicity tests and recovery experiments, certified dehydrated cysts of brine shrimp \(A.\ franciscana\) were purchased from the company MicroBioTests (Ghent, Belgium). Hatching of the cysts was obtained following the procedure described by Garaventa et al., 2010, by incubating 100 mg of cysts in glass Petri dishes containing NSW, for 24 h at 25 \(\pm 1^\circ\text{C}\) under light source (3000–4000 lx).

Newly hatched brine shrimp larvae (Instar I nauplius stage) were separated from unhatched cysts and transferred based onphototaxis into new glass Petri dishes with NSW.

2.2.1. Acute toxicity test

Acute toxicity tests (see Table 2) were performed according to standard APAT IRSA CNR 8060 method (CNR, 2003), by adding 10 nauplii to each well of 24-well plates, containing 2 ml with suspensions of different concentrations of the PS NPs tested (0, 5, 10, 25, 50, and 100 \(\mu\)g/ml) in NSW.

Control was settled in NSW without PS NPs. The plates were kept at 25 \(\pm 1^\circ\text{C}\) for 48 h in dark conditions, without providing food according to Garaventa et al. (2010). Potassium dichromate was tested as reference toxicant.

At 24 and 48 h, the number of dead nauplii (which were motionless for 10 s) was counted under stereomicroscope, in order to calculate the mortality. The validity of the test was guaranteed by the control group showing < 10% of mortality at 48 h. Moreover, at 48 h nauplii were also observed by optical fluorescence microscopy in order to identify any potential sub-lethal effects (see Table 2) (i.e. molting, PS NPs accumulation in the digestive tract or adhesion to the external appendages). A tentative method for calculating the amount of molts released by developing larvae was developed as follow: media from experimental groups (control-NSW and larvae exposed to PS-NPs-NSW) were collected, filtered through a 70 \(\mu\)m Falcon Cell Strainer Nylon, which retained all larvae and then rinsed several times with NSW. All media were then centrifuged at 12000 rpm for 5 min and the obtained pellet weight and compared with control exposed media. This method will allow the separation of larvae from molts which were quantified by gravimetry. A number of 400 larvae were considered for the quantification of the amount of molts.

All the experiments have been performed in triplicates and repeated at least three times. In order to determine the presence of PS NPs in the digestive tract of nauplii brine shrimp larvae were observed under optical fluorescent microscope Olympus BX51 (filter FITC 470/525 for PS-COOH; filter DAPI 365/445 for labelled PS-\(\text{NH}_2\)). Images were taken with DP50 camera at 10 \(\times\) using Olympus DP-software.

2.2.2. Recovery experiment

A recovery experiment was performed following the procedure described by Ates et al. (2013a). Brine shrimp larvae (Instar II-III nauplius stage) after 48 h of exposure were collected by a Pasteur pipette, rinsed using a 100 \(\mu\)m Falcon cell strainer and transferred to 6-well plate, containing 6 ml of NSW without PS NPs. In these conditions, nauplii were allowed to deparate at 25 \(\pm 1^\circ\text{C}\) for 24 h in the dark. No food was provided during the recovery test. After 24 h of recovery, brine shrimp larvae were examined under optical fluorescent microscope to determine gut clearance and removal of any PS NPs on the external surface and appendages of the earlier exposed larvae, compared to the control group. Recovery experiments have been performed in triplicates and repeated at least three times.

2.3. Data analysis

All statistical analysis were performed using Graphpad Prism5. Analysis of variance (ANOVA) was performed to compare the various treatments, and \(p < 0.05\) was taken as significant cut-off. Results of acute and recovery tests are mean of at least three independent experiments. LC_{50} values were calculated by fitting the percentage of alive larvae to a classical sigmoidal dose-response model according to the equation: \(y = b + (a - b) / 1 + 10^{(\log EC_{50} \times x)}\) where \(y\) is response, \(b\) response minimum, \(a\) response maximum, \(x\) the logarithm of effect concentration and LC_{50} the concentration of effect giving 50% of maximum effect. Each experiment has been performed 3–5 times.

3. Results and discussion

3.1. PS NPs and behavior in exposure media (natural sea water)

Primary particles nominal size of 40 nm PS-COOH and 50 nm PS-\(\text{NH}_2\) was confirmed by TEM imaging (Fig. S1).

DLS showed the formation of large PS-COOH aggregates in NSW at 25 \(\pm 1^\circ\text{C}\) with a \(Z\)-average larger than 0.9 \(\mu\)m, while PS-\(\text{NH}_2\) resulted far less aggregated with a \(Z\)-average of 106 nm.
(± 2 nm s.d.) and a PDI of 0.24 (Table 1). ζ-potential measurements confirmed their anionic (−9 mV) and cationic (+18 mV) surface charges (though the presence of aggregation in the PS-COOH NPs can affect these values). The low absolute values also indicated low stability in the NSW media. Both PS NPs showed an increasing aggregation in NSW with time (0 until 48 h): for PS-NH₂, PDI from 0.24 to 0.4 while for PS-COOH remains > 0.3 (Table 1). The observed low stability might be related to the high ionic strength of the NSW, which can screen the particle surface charges leading to the observed aggregation, unless the particles are stabilized by other factors, such as for instance adsorption of biomolecules on their surface (Corsi et al., 2014). A confirmation is given by the low absolute values of ζ-potential measured for both PS NPs in NSW which suggests a screening effect of surface charges due to the higher salt content but also by proteins or other compounds in the surroundings as for instance the natural organic matter (NOM) present in NSW (Table 3) (Wang et al., 2013b). NOM as well as natural mineral remain suspended in seawater as bio- and geogenic colloids being able to interact with NPs. The so-called heteroaggregation phenomenon is driven by the affinity between the high surface energy (e.g. charges) of the NPs and these naturally occurring colloids in NSW (Corsi et al., 2014).

Our findings underline the need to deeply characterize stability of NPs in complex environmental media as NSW, which therefore is recommended to be used in standardized ecotoxicity tests. A combination of parameters such as pH, ionic strength, salt concentrations and the presence of other biomolecules, similarly to what observed for proteins forming a corona on the NP in human blood, might strongly affect the behavior of surface charged NPs as PS in the media and more important their interactions within cells (Wang et al., 2013b).

Based on our findings, nanoscale aggregates of PS-NH₂ (~100 nm) are still present in NSW media while PS-COOH NPs originated microscale aggregates (> 900 nm) (Table 1).

3.2. Brine shrimp *A. franciscana* larvae acute toxicity test

In order to assess acute toxicity of anionic and cationic PS NPs (40 nm PS-COOH and 50 nm PS-NH₂ respectively), brine shrimp larvae were exposed to NPs suspension in NSW for 48 h and observed for mortality and NPs accumulation according to the standard APAT IRSA CNR 8060 method (CNR, 2003) (Fig. 1a and b).

No significant mortality was observed at 48 h (naplius stage) for both PS NPs up to 100 μg/ml tested concentration but several sub-lethal effects were evident (Table 2).

Light microscopy images of larvae at 12 h of exposure clearly showed uptake of PS-COOH aggregates at all tested concentration (5–100 μg/ml), absent in controls. A massive sequestration inside the gut lumen was evident for both PS NPs (5–100 μg/ml) at 48 h (nauplius stage), as shown in Fig. 1(a and b). A further confirmation of the nature of aggregates was given by fluorescent microscopy which revealed green fluorescence in the gut of PS-COOH exposed larvae as well as blue fluorescence of PS-NH₂ NPs exposed ones (Fig. 1c and d).

By continuous recording after 48 h of exposure, some of them were lately excreted as fecal pellets but not a full release of aggregates present in the gut lumen was observed (see Fig. S2 and the video on the Supplementary Information). This peculiar uptake and sequestration behavior of NPs observed in brine shrimps have been recently described for several NPs (Arulvasu et al., 2014; Ates et al., 2013a, 2013b; Auffan et al., 2013; Gambardella et al., 2014; Pretti et al., 2014; Rodd et al., 2014) as well as for PS micro-beads in other zooplankton species (Cole et al., 2013; Lee et al., 2013). Our recent paper on sea urchin embryos was the first contribution showing the accumulation of PS-COOH NPs in embryos during development (Della Torre et al., 2014a). Accumulation seems in general not affecting mortality but being more associated with several sub-lethal effects (i.e. behavioral, physiological and biochemical) which can affect survival of brine shrimp in prolonged exposure scenarios. As already hypothesized for microplastics, the observed accumulation of PS NPs aggregates in the digestive tract may limit food intake and significantly affect growth and development of brine shrimp larvae (Besseling et al., 2014; Cole et al., 2013). In addition, the documented high ability of PS nano-debris to adsorb hydrophobic toxic contaminants may increase their bioavailability and consequently toxicity to marine organisms (Rochman et al., 2013; Velzeboer et al., 2014). More studies regarding this phenomenon are urgently needed based on the evidence of significant gut accumulation of PS NPs in exposed marine organisms as in general of other NPs as well. Our recent studies using titanium dioxide NPs provide first insight concerning the potential interference between NPs and toxic marine pollutants with consequences at various level from increase accumulation of toxicant (e.g. coupled with dioxin) to decrease toxicity (e.g. cadmium) (Canesi et al., 2014; Della Torre et al., 2014b, 2015).

In more realistic scenarios as during chronic exposure in the natural environment, bioavailability and uptake of both anionic and cationic PS NPs by planktonic species could lead to their transfer along marine trophic web with significant ecological consequences being zooplankton an important food source for other marine organisms. Moreover, the excretion of nano-sized polymers as PS NPs in fecal pellets could enhance their removal from the sea surface by increasing their sinking at the sea bottom level (Cole et al., 2013).

Despite PS-NH₂ (5–100 μg/ml) also accumulated alike in brine shrimp swimming larvae at 48 h, many specimens showed empty digestive traits (Fig. 1d and e). More clearly, these cationic nanoplastics were stuck to the external surface of the swimming larvae (Fig. 2c) and in particular to the sensorial appendages, as shown in Fig. 2c and d. The presence of PS-NH₂ on the appendages was found to noticeably hampering brine shrimp larvae natation at 48 h and thus probably limiting their ability of feeding. Brine shrimp *A. franciscana* creates feeding currents while swimming in order to ingest waterborne particles (Ruppert et al., 2004). Therefore longer exposure scenarios will aim to evaluate the outcome of these sub-lethal observed effects and predict possible consequences at organism and population level by disrupting behavior, feeding and growth.

Cole et al. (2013) recently highlighted that, depending on the size, microplastics can be ingested (7–20 μm) or externally adhere

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**Table 1**

Physico-chemical characterization of PS NPs in Milli-Q water (mQW) and natural sea water (NSW) (0.45 μm filtered, T = 25 ± 1 °C, salinity 38%, pH 8.3, conductivity 6.5/m) using DLS analysis showing Z-average (nm), polydispersity index (PDI) and ζ-potential (mV). Data are referred to PS NPs concentration of 50 μg/ml and values reported as average ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>40 nm PS-COOH</th>
<th></th>
<th>50 nm PS-NH₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z-Average (nm)</td>
<td>PDI</td>
<td>ζ-potential (mV)</td>
</tr>
<tr>
<td>mQW</td>
<td>58 ± 2</td>
<td>0.129 ± 0.01</td>
<td>−34 ± 1</td>
</tr>
<tr>
<td>NSW &gt; 0.9 μm</td>
<td>0.302 ± 0.08</td>
<td>−9 ± 2</td>
<td>106 ± 2</td>
</tr>
</tbody>
</table>
(3.8 μm) to zooplankton. In our case, the different aggregation of the two PS NPs in NSW and not their size seems not fully explain the observed effects. PS-NH2 NPs which are quite well dispersed (106 nm) in NSW still accumulate to some extent in the digestive tract of brine shrimp larvae. Therefore, surface charge seems more responsible of the observed different interaction and impact to marine organisms, as already described in our previous study with sea urchin _P. lividus_ embryos (Della Torre et al., 2014a).

In addition, at the highest PS-NH2 concentrations (50 and 100 μg/ml), several molts were found in the NSW media after 48 h of exposure (Fig. 2d) compared to controls and lower concentrations. A tentative quantitative assessment of molts released by PS-NH2 exposed larvae was developed. A significant increase of around 50% of molts respect to controls was observed in PS-NH2 exposed larvae. This might represent a good tentative method for quantifying the molts events caused by NPs exposure since in anostracans molts are quite transparent and were easily broken by other brine shrimps swimming through. By the way, this effect has been observed several times in all replicates (10) and in the three parallel experiments performed. An increasing number of molts seemed also to be present at PS-NH2 NPs higher concentrations (50 and 100 μg/ml). This is the first observation of an increase of molting events in zooplankton species exposed to NPs and further investigations should focus on mechanisms able to disrupt this hormone-controlled physiological phenomenon, which is considered the most crucial step in the life cycle of microcrustaceans as brine shrimp. The importance of molting in the biodistribution and release of NPs has been described by Auffan et al. (2013) in _Daphnia pulex_ exposed to CeO2 NPs. While ingestion can be considered the major route of NPs uptake in microcrustaceans, ecdisis

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**Table 2**

Summary of the experimental design and biological effects observed in brine shrimp _A. franciscana_ larvae after exposure to PS-COOH and PS-NH2 NPs.

<table>
<thead>
<tr>
<th>PS NPs</th>
<th>Test</th>
<th>Reference</th>
<th>Concentrations</th>
<th>End-point</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 nm PS-H</td>
<td>Acute toxicity test (48 h)</td>
<td>APAT IRSA CNR 8060 (2003)</td>
<td>0, 5, 25, 50, and 100 μg/ml</td>
<td>Mortality, Sub-lethal effects</td>
<td>None &lt; 100 μg/ml</td>
</tr>
<tr>
<td>40 nm PS-COOH</td>
<td>Recovery (24 h)</td>
<td>Ates et al. (2013a)</td>
<td>0, 5, 25, and 50 μg/ml</td>
<td>Gut clearance, Mortality, Sub-lethal effects</td>
<td>Not</td>
</tr>
<tr>
<td>50 nm PS-NH2</td>
<td>Acute toxicity test (48 h)</td>
<td>APAT IRSA CNR 8060 (2003)</td>
<td>0, 5, 25, 50, and 100 μg/ml</td>
<td>Adherence, molting</td>
<td>Removal from external surface</td>
</tr>
<tr>
<td>50 nm PS-NH2</td>
<td>Recovery (24 h)</td>
<td>Ates et al. (2013a)</td>
<td>0, 5, 25, and 50 μg/ml</td>
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**Fig. 1.** Effects of 40 nm PS-COOH and 50 nm PS-NH2 on brine shrimp _A. franciscana_ nauplii at 48 h: (a) control in NSW; (b, c) accumulation of green fluorescent PS-COOH (25 μg/ml) and (e, f) blue fluorescent PS-NH2 (25 μg/ml) inside the digestive trait; (d) detail of fecal pellet containing PS-COOH aggregates. Images are representative of three independent experiments. Scale bar: 100 μm.
(molting) has been considered as the main physiological mechanism of CeO₂ NPs release from Daphnia able also to decrease the direct trophic transfer to predators.

Based on our findings, we hypothesize that the increase of molting events in brine shrimp larvae may represent a defense mechanism regardless the exposure to cationic PS NPs (PS-NH₂). The potential link between an increase in molting and the presence of PS-NH₂ NPs aggregates adhering to the external surface and appendages of larvae might better explain the interaction between NPs and the exposed larvae and explain the mechanism behind the observed effects.

Molting is also energy consuming and according to Cole et al. (2014) copepods receive less energy for their metabolism and reproduction during this peculiar stage. An increase of molting events could therefore potentially affect this energy flow with serious consequences on brine shrimp larvae growth. Long-term study including also low levels of PS-NH₂ exposure will help to define how cationic nanoplastics may affect brine shrimp A. franciscana physiology and consequently survival and reproduction.

4. Recovery Experiment

As reported by previous studies (Ates et al., 2013b; Cole et al., 2013; Lee et al., 2013), the ingestion of synthetic micro-beads by zooplankton species can heavily hinder the digestive trait, thus limiting feeding, growth and survival. In order to understand the extent of this phenomenon, a recovery experiment was performed. After 48 h of exposure, brine shrimp larvae were left in clean NSW (no PS NPs) for 24 h without feeding and then observed by light and fluorescent microscopy.

All brine shrimp nauplii (10 organisms in each experiment) earlier exposed to PS-COOH (0, 5, 25, and 50 μg/ml) still presented aggregates in their gut, even at the lowest NPs concentrations, in agreement with the retention of microplastics up to 7 days observed by (Cole et al., 2013) in marine copepods. Moreover, the presence of food during the recovery experiments has been shown to improve the elimination efficiency of NPs from the digestive tract of brine shrimp A. franciscana, even if a significant proportion was be retained in the gut (Ates 2013a, 2013b). On the opposite, larvae exposed to PS-NH₂ (0, 5, 25, and 50 μg/ml) and transferred to clean NSW did not show neither aggregates in the gut nor NPs attached to the external surface and appendages. However, further studies are required to exclude any potential negative impact on brine shrimp larvae due to nano-PS exposure, since long-term exposures could provide in-depth information upon the effects of nanoplastics on brine shrimp A. franciscana.

5. Conclusion

Our study suggests that PS NPs might pose a risk to marine zooplankton as a result of exposure to nanoplastics at the concentrations tested here. Nano-sized PS might be able to impair food uptake (feeding), behavior (motility) and physiology (multiple molting) of brine shrimp larvae A. franciscana with consequences not only at organism and population level but on the overall ecosystem based on the key role of zooplankton on marine food webs. In addition, our study again underline that careful assessment of NP properties and stability in NSW is needed in order to properly address their behavior towards marine organisms. Aggregation but more important surface charges (cationic vs anionic) may lead to different uptake and biodistribution and perhaps disrupt important physiological function linked to feeding and growth as observed in our study. The European Marine Strategy Framework Directive has a key goal of ensuring that marine litter is kept to a level that causes “no significant harm to the marine environment” and hence there are strong links with

### Table 3

<table>
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<tr>
<th>Parameters</th>
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<tbody>
<tr>
<td>TOC</td>
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<tr>
<td>Total oxygen</td>
<td>6,6 mg/L</td>
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<tr>
<td>Cr</td>
<td>&lt; 1 μg/L</td>
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<tr>
<td>As</td>
<td>&lt; 1 μg/L</td>
</tr>
<tr>
<td>Cd</td>
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<tr>
<td>Hg</td>
<td>&lt; 1 μg/L</td>
</tr>
<tr>
<td>Pb</td>
<td>&lt; 1 μg/L</td>
</tr>
<tr>
<td>Total PAH</td>
<td>0,12 mg/Kg</td>
</tr>
</tbody>
</table>

Fig. 2. Effects of 50 nm PS-NH₂ on brine shrimp A. franciscana nauplii at 48 h. Upper images showing control in NSW: detail of clear (a) sensorial antennules, (b) antennae and (c) abdomen. Lower images showing PS-NH₂ (50 μg/ml) exposed nauplii (48 h): detail of nanoplastics attached to (d) sensorial antennules, (e) sensory hairs of the antennae and (f) abdomen region. Aggregates of PS-NH₂ are indicated by black arrows. Images are representative of three independent experiments. Scale bar: 100 μm.
the development of sound environmental policy to meet this need at global level including to assess the impact of nano-debris as nanoplastics in the marine environment.

Acknowledgments

This study was performed in the framework of the PhD project entitled “Polystyrene nanoparticles and their impact on marine ecosystems: accumulation, disposal and toxicity in marine species from Antarctic and Mediterranean Seas”. PhD student Elisa Bergami, PhD School in Geological, Environmental and Polar sciences and technologies, Department of Physical, Earth and Environmental Sciences, University of Siena (Italy). Funded by the University of Siena and the Italian National Antarctic Museum “Felice Ippolito”.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2015.09.021.

References


Fig. 3. Two examples of molts found in wells containing brine shrimp A. franciscana nauplii after 48 h of exposure to 50 nm PS-NH2. Anostracans such as A. franciscana are characterized by absence of carapace, therefore molts appeared quite transparent and easily broken by other brine shrimps swimming through. 10× images are referred to 50 μg/ml exposed organisms and representative of three independent experiments. Scale bar: 200 μm.

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waters to freshwater and marine organisms.


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