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

Elsevier Editorial System(tm) for Ecotoxicology and Environmental Safety
Manuscript Draft

Manuscript Number: EES-15-605R1

Title: Nano-sized polystyrene affects feeding, behaviour and physiology of brine shrimp Artemia franciscana larvae

Article Type: SI: BECOME Meeting

Section/Category: Ecotoxicology

Keywords: Nanoplastics; polystyrene; marine zooplankton; Artemia franciscana; accumulation; molting

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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 naplius stage but several sub-lethal effects were evident. PS-COOH (5-100µg/ml) resulted massively sequestered inside the gut lumen of larvae (48h) 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 (48h) but also adsorbed at the surface of sensorial antennules and appendages probably hampering larvae motility. In addition, larvae exposed to PS-NH2 undergo multiple molting events during 48h 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 48h 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.

*Cover Letter

Università degli Studi di Siena
Dipartimento di Scienze Fisiche, della Terra e dell'Ambiente

Siena, 6th September 2015

Dear Editor

Please find enclosed the electronic submission of our revised manuscript entitled:

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

The main subject of the manuscript is to investigate distribution and sub-lethal effects of carboxylated (PS-COOH) and amine ($PS\text{-}NH_2$) polystyrene nanoparticles as model nanoplastics in brine shrimp *Artemia salina* in order to determine whether these materials have similar effects in marine organisms as what observed in common human and other mammalian cell lines.

Nanoplastic debris, resulted from run-off and weathering breakdown of macro and microplastics, represent an emerging concern for marine ecosystems. While microplastics are quite well studied, fate and impact of nanoscale plastics in the marine environment is almost unknown and this is raising concern due to the increasing abundance in water column and food webs and nanoscale properties which could imply toxicity to marine biota. Polystyrene NPs can be considered as good model for studying both environmental fate of nanoplastics, in terms of interactions with the surrounding media, and toxicity for marine organisms focusing on specific pathways of cellular uptake. In fact the toxicity mechanisms have been quite well described in human cell models, while how PS NPs can interact with their surroundings as for instance with marine waters and enter the cells of marine organisms is still largely unknown.

Nanoparticle stability in natural seawater was measured by DLS, while distribution and toxicity (mortality) were monitored through light and fluorescence microscopy within 48 h of exposure.

The detailed secondary characterization performed in the present study clearly showed that in natural seawater brine shrimp larvae are exposed mainly to nanoscale objects in the case of $PS\text{-}NH_2$ (< 100 nm) while PS-COOH NPs originated microscale aggregates (> 1000 nm).

Our findings suggest that the different aggregation of the two tested PS NPs in natural sea water (89 nm for PS-NH_2 and PDI > 0.4 for PS-COOH) and, more importantly, the different surface charges might affect their cellular uptake and distribution and consequent sub-lethal effects in brine shrimp larvae.

Our results showed that brine shrimp larvae might be vulnerable to the amino modified PS NPs, as observed for mammalian cell lines and confirmed also on our recent work on sea urchin embryos (Della Torre et al., 2014). PS-COOH NPs did not showed any relevant effect but a significant accumulation inside the gut (as well as PS NH_2) was observed and this may also determine transfer through the trophic food web, raising serious concern about the exposure of organisms at higher trophic levels.

Furthermore, our study also show that careful assessment of NP properties and stability in natural sea water media is needed in order to properly determine their characteristics once exposed to marine organisms (e.g. plankton), as different aggregation state may lead to different uptake and distribution routes, and the different surface properties clearly have different impact.

We hope that the revised version of the ms could comply with the standards of Ecotoxicology and Environmental Safety journal and will be considered for publication in the special issue: BECOME meeting. The authors state that there is no conflict of interest.

Many thanks in advance for your consideration.

Your Sincerely

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Reviewer suggestions

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*Response to Reviewers

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Università degli Studi di Siena

Dipartimento di Scienze Fisiche, della Terra e dell'Ambiente

Siena, 6th September 2015

Journal: Ecotoxicology and Environmental Safety

Ms. Ref. No EES-15-605

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

Author(s): Elisa Bergami, Elena Bocci, Maria Luisa Vannuccini, Marco P. Monopoli, Anna Salvati, Kenneth A Dawson, Ilaria Corsi

Dear Editor,

We do thank you and the two Reviewers for their comments and criticisms.

The manuscript (ms) had been modified accordingly following the comments.

Reviewer #3: From my point of view this paper can be presented as original research article because overall the obtained results fall to reach the purposes indicated by the authors: i) evaluate the effects of 40 nm anionic carboxylated (PS-COOH) and 50 nm cationic amine (PS-NH2) polystyrene nanoparticles on brine shrimp Artemia larvae by acute mortality test; ii) evaluate the uptake and distribution of these NPs on the brine shrimp, also observing sub-lethal effects during the mortality test.
In my opinion, the original finding of this paper is the observation of accumulation, excretion, adherence at the surface of sensorial antennulae and appendages, and molting induction.

I evaluate positively the tentative quantitative assessment of molts released by PS-NH2 exposed larvae, by separation of larvae from molts which were quantified by gravimetry.

In addition, physico-chemical characterization of PS-COOH and PS-NH2 NPs is also reported using Dynamic Light Scattering analysis and showing Z-average (nm), polydispersity index (PDI) and <zeta>-potential (mV).

We thank the reviewer for having appreciated the manuscript and the quality of the data presented.

The manuscript is well written and I agree to accept it with minor revisions as follow:

- In general: in many parts of the text spaces between words are missing and sometimes the verb is not correctly included in the singular or plural form.

All spaces have been inserted and verbs have been checked carefully and corrected accordingly.

- Lines 194-195: Artemia salina species is reported in the text. Do authors want to refer to Artemia franciscana?

Thanks to advices provided by zoologists experts on Artemia genus, we now are confident in indicating the Artemia larve used in our study as franciscana and not salina as previously stated. So both in the title and in the ms the species has been changed from salina to franciscana. We also decided to include the word "larvae" in order to make more clear the biological model used.
We do thanks the reviewer for such criticism since perhaps we did not checked properly the origin and attributes of the larvae used in the study.

- Lines 195-196 "were purchased from Laboratory … " It should be "… from the company MicroBioTests (Ghent, Belgium)"

It has been changed according to reviewer suggestion.

- Lines 226, 285, Recovery experiment: has it been carried out according to Ates et al (2013a) (as reported at line 227) or to Auffan et al (2013) (as reported in table 2)?

Thanks, there was a mistake in the citation, it has been now changed as Ates et al 2013a

- Line 221-222, table 2, Accumulation analysis: has it been carried out according to APAT IRSA CNR 2003 (as reported in Table 2) or by optical fluorescent microscope (as reported at lines 221-222)? I seem that this method is only for the mortality test and it doesn't provide an accumulation outcome. Has accumulation been only observed or quantified?

The reviewer is right, the cited method is only a mortality test and this is what we meant in Table 2 by including this citation. Based on the strong accumulation we previously observed in another model organism as sea urchin (see Della Torre et al., 2015) we decided to observed brine shrimp larvae under optical fluorescence microscopy. We are now working on a protocol for quantify the amount of fluorescence in order to get a dose-response curve and perhaps to cite in our future ms.
In order to clarify which method has been used for mortality and accumulation, we have inserted the following sentence in the text after line 211.
"Moreover, at 48 h nauplii were also observed by optical fluorescence microscopy in order to identify any sub-lethal effects (see Table 2) including molting, the presence of PS NPs accumulated in the digestive trait or adhering to the external appendages."

- Line 322: verify "in press"

It has been changed as Della Torre et al., 2015.

- Lines 377-380, figure 1 : I don't see image d)

The "d" image is inside the "c" as spot in the corner.

Reviewer #1: The authors intends to show the importance of the effects provoked by the presence of nano-plastics in the environment, by using two differently charged polystyrene NPs. Despite the importance of the observation and the study of these NPs in particular (most probably of high importance in a very near future), is missing the physicochemical characterization of the natural waters samples on the Tuscan archipelago, which will undoubtedly help on the understanding of the effects observed. So, I advise the authors to show this characterization and use it to discuss the effects obtained. Even, because the authors say at least twice that is very important to understand the properties of the particles when dispersed in the medium used for the toxicity tests.

We thank the reviewer for such criticism, a new Table indicated as "Table 3" has been included in the ms showing physico-chemical parameters of natural sea water (NSW) samples used in the present study including also selected contaminants.

Beside this main comment some other major comments need to be clarified before publication.

1) Numerous type errors can be found through all text; most of them related with the absence of a space between words. Some examples are: 4th highlight "PS-NH2 were…"; Abbreviations "… (PS-COOH), amino…"; Line 69 "… 2014). Concerning…", …

All ms has been checked carefully and all type-errors corrected accordingly. We realized that this problem might be probably related to have opened the file with two different version of Microsoft Word program.

2) Line 72. Should read "… sized, since EU…" and not "… sized in this area, since EU…"

Thanks, changed.

3) Lines 72-73. "Good environmental status". This is very vague, doesn't mean a lot.

The main goal of the European Marine Directive (Directive 2008/56/EC) is to achieve Good Environmental Status of EU marine waters by 2020. The Directive defines Good Environmental Status (GES) as:

"The environmental status of marine waters where these provide ecologically diverse and dynamic oceans and seas which are clean, healthy and productive" from Article 3 of Directive 2008/56/EC.

In order to better explain such citation, the sentence has been rewritten and the Directive citation included.

4) Lines 80-81. The authors could give the measured/expected quantities and also the intrinsic properties that make these particles important to be studied.

Unfortunately there are no data available regarding neither measured nor expected quantities of polystyrene nanoparticles in sea water samples. Please refer to the ms of Besseling et al., 2014 (cited) in which a sort of assessment has been done but only in freshwater and not in sea water. Concerning the intrinsic properties please refer to the several papers of co-authors of the ms, Monopoli and Dawson, cited in the introduction from line 111 to 119.

5) Line 86. Should read "… expected that they would be more severely exposed to nano-sized… and not "… expected to be more severely exposed even to nano-sized…

Many thanks for the advice, it has been changed

6) Lines 89-90. This sentence is very confuse and I really cannot understand what the authors want to say with this.

Thanks for the criticism, the sentence has been rewritten in two new sentences better explaining the concept of behavior of PS NPs in sea water column as consequence of aggregation.

7) Line 94. “selected” why not to give the composition of the NPs?

The composition has been included in the lines 111-114 of the ms.

8) Lines 98-119. Both paragraphs should be after the sentence on line 81.

Thanks for the criticism, the sentences have been moved after line 81.

9) Line 125. Should read “… embryos, which were the first evidence of toxicity reported…” and not “… embryos, where also first evidence of toxicity were reported…”

Thanks. Changed.

10) Line 131. What are the standard PS?

We perhaps used the word “standard” not properly, we referred to unmodified PS NPs, without surface functionalization.

11) Line 132. Should read “… determined yet.” And not “… determined as yet.”

Thanks. Changed.

12) Lines 132-134. What the authors meant with this? Is necessary to rewritten this sentence.

Thanks, it was not well written since the verb was in the wrong position. It has been rewritten.

13) Line 147. Should read “The aim of the present…” and not “The aim of present…”

Thanks. Changed.

14) Lines 147-149. It would make more sense to use the exact same size of the NPs in order to evaluate only the effect of the charge. When thinking in surface area 10 nm in difference is big and may cause very different effects; what is the difference in the quantity of groups at the surface of the particle?

To our knowledge , the only available labeled PS-NPs are those of 40 nm PS-COOH and 50 nm PS-NH2. Past studies on human models tested the same PS-NPs (see cited papers of co-authors Dawson and Monopoli) and such slight difference in dimension has not been considered so important in term of observed toxicity. Being aggregation quite different between the two in NSW, we assume that even being different in primary dimension, once in NSW this difference may not play a significant role in term of groups at the surface of the aggregates. Nevertheless, we will keep this interesting suggestion for future studies.

15) Lines 163-169. In the way that is written it seems that is important to study these nano-plastics because they are NOT present in the environment! So, why to accept this manuscript, if it doesn’t have environmental relevance? This needs to be rewritten.

Thanks the suggestion, the sentence has been rewritten as follows: "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".

16) Lines 176-179. As mentioned above, is necessary to characterize these waters in terms of organic and inorganic content and metals quantities; the toxic effects can come from the presence of metals.

As mentioned above a new Table listing physico-chemical parameters including levels of metals of natural sea water samples used in the study has been included in the ms.

17) Lines 249-250. The TEM images shows that the sizes of the particles are not so similar; so, its important to give the average size and the standard deviations; also, the number of particles counted and in how many pictures.

The average size value of the particles has been included in legend of Figure S1. Regarding their measure it has been calculated using at least 10 differences pictures from each particles and by calculating the average values.

18) Lines 249-250. Why the TEM analyses were not performed for the NPs dispersed in the natural waters? (so, the exact same conditions where the tox experiments were performed.) Moreover, as the authors surely know that the DLS have a bias through larger particles proportional to d^6. So, its important that the aggregation obtained by DLS would be confirmed by TEM, and even because it seems that the authors have access to this instrument.

The TEM images were performed in both MilliQ water for primary characterization as well as in NSW for DLS data confirmation. Unfortunately, the background noise of NSW probably due to high ionic salts and NOM made almost impossible to recognize the shape of the PS-NPs and more important to measure their dimension. Due to this very low quality of TEM images and being useless for our study, we decided not to include them in the ms. Meanwhile, we are currently working to solve this issue but it seems a very difficult task for this specific type of NP. We published other papers on titanium dioxide NPs in which we easily showed good TEM images obtained in NSW samples of the same geographical origin (Della Torre et al., doi10.1016/j.hazmat.2015.04.072; Canesi et al. doi10.1016/j.aquatox.2013.11.002).

19) Lines 260-263. How the authors can affirm this if they don't show the characterization data of the natural waters? Also, this explanation will be dependent on the organic matter content.

Our hypothesis is based on recent studies in which the NOM present in NSW has been shown to drive heteroaggregation phenomenon of surface charged NPs so potentially affecting also the observed aggregation of PS NPs in NSW observed our study. The total organic carbon content listed now in Table 3 show a moderate level of organic matter in NSW samples used in or study so confirming such hypothesis and in agreement with other studies (please see Wang et al., 2013b, cited in the ms).

20) Lines 268-273. This paragraph is totally redundant since the authors didn't do this full characterization and even don't give the characteristics of the water; also, most of this is repeated in the paragraph above.

Again we were referring to data showed by Wang et al. 2013b but based on our revision and data included in the new Table 3, a confirmation of a role of such parameters in the behavior of such NPs in NSW has been given. The sentence from 271-273 is just describing the different dimension of PS-COOH and PS-NH2 aggregates observed in NSW.

21) Table 2 is never called in the main text body.

It has been called in line 202.

22) Line 301. During how much time this recording was done?

The recording was done for the further 24 h (recovery test).

23) Line 302. Should read "… (see Figure S2 and the video on the Supplementary…" and not (see Supplementary…"

Thanks. Changed.

24) Lines 317-318. Why this was not done within this study? It seems feasible.

This is the aim of the running experiments which are now about to end and will be published hopefully soon.

25) Lines 319-321. What this has to do with the issue being described and discussed in all this paragraph?

This sentence aim to address the ecological impact of potential effect of nanoplastics in planktonic species and the consequent impact on marine ecosystem due their key role in trophic web.

26) Lines 340-341. Please, rephrase this sentence.

The sentence has been rewritten.

27) Lines 351-353. This is said in the caption of Figure 3, and in fact it should be only here.

It has been deleted from the text.

28) Line 353. Should read "… several times… " and not "… several time…"

Thanks. Changed.

29) Line 354. Should read "… and in the three…" and not "… and the three…"

Thanks. Changed.

30) Line 358. Should read "… crucial step in the…" and not "… crucial in the…"

Thanks. Changed.

31) Line 404. So, why these studies were done in absence of food? Also, if there is already evidences that perhaps is necessary to wait more time, why these studies were done only during 24 h?

Based on our experience and in agreement with previous studies (see Garaventa et al., 2010), 24 h without food at such developmental stage is the maximum time allowed in order not to affect organism survival. Moreover, in order to maintain the same experimental media used in the acute test (48h) we decided not to add any food in the media which could potentially affect also PS NPs *behaviour by for instance increasing organic matter content. A long-term toxicity test with food is currently under investigation and data will be ready soon.*

32) Lines 406-408. Any idea why this would happen? Maybe some heteroagglomeration with organic or inorganic colloids present in the water (which cannot be attested since no detail characterization of the water is given)?

Based on the new data showed on Table 3 on NSW samples, such behaviour might be due to a better dispersion in NSW of positively charged PS NPS as PS-NH2 than negatively ones PS-COOH) in accordance with the hypothesis of heteroaggregation as reviewer also suggest.

We do hope that changes made following the suggestions of the reviewers have improved the quality of the manuscript so as to allow the publication in Ecotoxicology and Environmental Safety journal.

The authors state that there is no conflict of interest.

Your Sincerely

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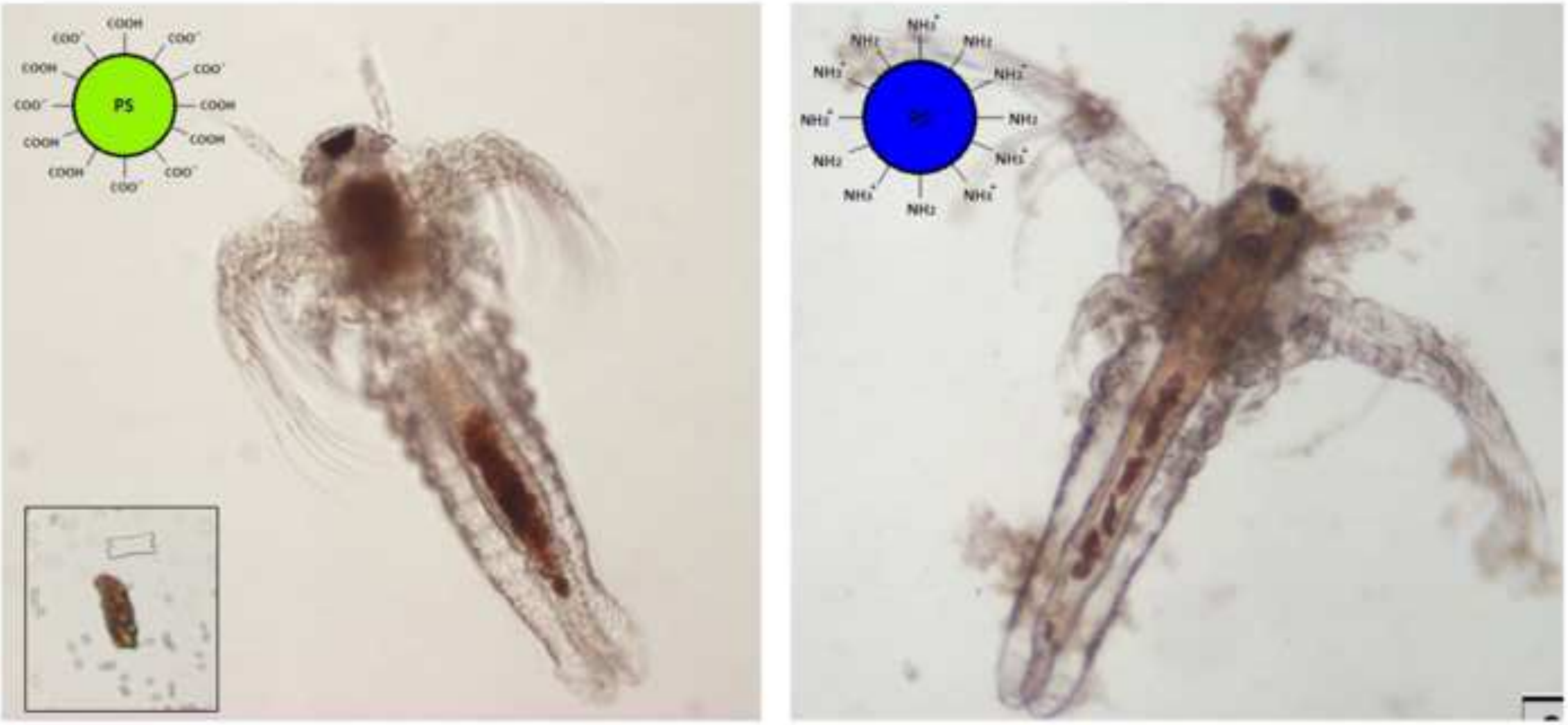
Graphical Abstract (for review)



Accumulation and excretion

Adherence and molting induction

Highlights

- Nowadays very little knowledge is available on the impact of nano-sized plastics on marine organisms.
- Polystyrene NPs (PS-COOH and PS-NH_2) caused no mortality at 48 h of exposure in larvae of brine shrimp *A. franciscana larvae*, but several sub-lethal effects were observed.
- PS NPs resulted massively sequestered inside the gut lumen of larvae (48 h), probably limiting food intake.
- PS-NH_2 were adsorbed at the surface of sensorial antennulae and appendages.
- Multiple molting events during 48 h of exposure compared to controls were observed upon exposure to PS-NH_2.

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-NH_2) 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 naplius stage but several sub-lethal effects were evident. PS-COOH (5-100µg/ml) resulted massively sequestered inside the gut lumen of larvae (48h) probably limiting food intake. Some of them were lately excreted as fecal pellets but not a full release was observed. Likewise, PS-NH_2 (5-100 µg/ml) accumulated in larvae (48h) but also adsorbed at the surface of sensorial antennules and appendages probably hampering larvae motility. In addition, larvae exposed to PS-NH_2 undergo multiple molting events during 48h of exposure compared to controls. The activation of a defense mechanism based on a physiological process able to release toxic cationic NPs (PS-NH_2) from the body can be hypothesized. The general observed accumulation of PS NPs within the gut during the 48h 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.

Keywords

Nanoplastics, polystyrene, zooplankton, *Artemia franciscana larvae*, accumulation, molting

Abbreviations

Polystyrene Nanoparticles (PS NPs), carboxylated (PS-COOH), amino (PS-NH_2), Natural Sea Water (NSW), Natural Organic Matter (NOM)

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 is 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 nano-sized 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 (Bandyopadhyay 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 nano-sized 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, 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 (-NH_2) (Casado et al., 2013; Loos et al., 2014). Several studies revealed their cellular uptake using *in vitro* models with human cell lines (Bramini, 2014; Lesniak et al., 2010; Liu et al., 2011; Lunov, 2011; Rossi et al., 2014), but also cytotoxicity and apoptosis in particular for PS-NH_2 (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 nano-sized 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, 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 of 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-NH_2 (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, 2008; Moore, 2006). Although commonly applied as model positively charged NPs in nanosafety studies, it is important to stress that PS-NH_2 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. CeO_2, 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; Ates et al., 2013b; Auffan et al., 2013; Gambardella et al., 2014; Pretti 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-NH_2) 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-NH_2 NPs

40 nm green carboxylated polystyrene nanoparticles (PS-COOH) (505 nm excitation, 515 nm emission) were purchased from Invitrogen. Unlabeled and blue fluorescently labeled (358 nm excitation, 410 nm emission) 50 nm amino modified polystyrene (PS-NH_2) 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; Wang et al., 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-NH_2 were also considered as positively charged nanoplastics, although they are a special surface functionalised 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 (ζ-) potential (mV) (Zetasizer Nano Series software, version 7.02, Particular Sciences, UK). Measurements have been performed in triplicate, each containing 11 runs of 10 seconds for determining Z-average, 20 runs for the ζ-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 μm filtered NSW (T 25±1°C, salinity 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; Salvati 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-NH_2 caused apoptosis in 1321N1 human cells at 50 µg/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 µg/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 Persoone, 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 48h in clean NSW and left there for 24h. 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±1°C under light source (3000-4000 lux)

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, 100 µg/ml) in NSW.

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

At 24 and 48h, the number of dead nauplii (which were motionless for 10 seconds) 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 trait 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 throught a 70 µm 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 minutes 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 trait 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-NH_2). Images were taken with DP50 camera at 10X 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 48h of exposure were collected by a Pasteur pipette, rinsed using a 100 µm Falcon cell strainer and transferred to 6-well plate, containing 6 ml of NSW without PS NPs. In these conditions, nauplii were allowed to depurate at 25±1°C for 24 h in the dark. No food was provided during the recovery test. After 24h 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: $y = b + (a - b) / 1 + 10^{(\mathrm{Log\ EC50} - x)}$ 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 to 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-NH_2 was confirmed by TEM imaging (Fig. S1).

DLS showed the formation of large PS-COOH aggregates in NSW at 25±1°C with a Z-average larger than 0.9 µm, while PS-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-NH_2, 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, 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 biogenic 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, 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-NH_2 (~ 100 nm) are still present in NSW media while PS-COOH NPs originated microscale aggregates (> 900 nm) (Table 1).

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 S/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.

	40 nm PS-COOH			**50 nm PS-NH_2**		
	Z-Average (nm)	**PDI**	**ζ-potential (mV)**	**Z-Average (nm)**	**PDI**	**ζ-potential (mV)**
mQW	58 ± 2	0.129 ± 0.01	- 34 ± 1	61 ± 0.2	0.131 ± 0.02	+ 24 ± 1
NSW	> 0.9 µm	0.302 ± 0.08	- 9 ± 2	106 ± 2	0.243 ± 0.01	+ 18 ± 10

Table 2. Summary of the experimental design and biological effects observed in brine shrimp *A. franciscana* larvae after exposure to PS-COOH and PS-NH_2 NPs.

PS NPs	**Test**	**Reference**	**Concentrations**	**End-point**	**Outcome**
40 nm PS-COOH	Acute Toxicity Test (48 h)	APAT IRSA CNR 8060 (2003)	0, 5, 25, 50, 100 µg/ml	Mortality	None < 100 µg/ml
				Sub-Lethal Effects	Accumulation
	Recovery (24 h)	*Ates et al., 2013a*	0, 5, 25, 50 µg/ml	Gut Clearance	Not
50 nm PS-NH_2	Acute Toxicity Test (48 h)	APAT IRSA CNR 8060 (2003)	0, 5, 25, 50, 100 µg/ml	Mortality	None < 100 µg/ml
				Sub-Lethal Effects	Adherence Molting
	Recovery (24 h)	*Ates et al., 2013a*	0, 5, 25, 50 µg/ml	Removal from external surface	Yes

Table 3. Physico-chemical parameters including heavy metals (Cr, As, Cd, Hg, Pb, total polycyclic aromatic hydrocarbons, PAHs) of natural sea water samples used in the study. Data also available at: SIRA RSS. www. sira.arpat.toscana.it

	Parameters
TOC	1,3 %
Total oxygen	6,6 mg/L
Cr	< 1 µg/L
As	1 µg/L
Cd	0,09 µg/L
Hg	0,02 µg/L
Pb	< 1 µg/L
Total PAH	0,12 mg/Kg

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-NH_2 respectively), brine shrimp larvae were exposed to NPs suspension in NSW for 48h and observed for mortality and NPs accumulation according to the standard APAT IRSA CNR 8060 method (CNR, 2003) (Figure 1a,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 12h 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 Figure 1 (a, 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-NH_2 NPs exposed ones (Figure 1 c,d).

By continuous recording after 48h 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 Figure 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; Ates et al., 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 trait 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, 2015; Della Torre, 2014b).

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-NH_2 (5-100 µg/ml) also accumulated alike in brine shrimp swimming larvae at 48 h, many specimens showed empty digestive traits (Fig. 1d,e). More clearly, these cationic nanoplastics were stuck to the external surface of the swimming larvae (Figure 2c) and in particular to the sensorial appendages, as shown in Figure 3 c,d. The presence of PS-NH_2 on the appendages was found to noticeably hampering brine shrimp larvae natation at 48h 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 (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-NH_2 NPs which are quite well dispersed (106 nm) in NSW still accumulate to some extent in the digestive trait 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-NH_2 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-NH_2 exposed larvae was developed. A significant increase of around 50% of molts respect to controls was observed in PS-NH_2 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-NH_2 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 CeO_2 NPs. While ingestion can be considered the major route of NPs uptake in microcrustaceans, ecdysis (molting) has been considered as the main physiological mechanism of CeO_2 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-NH_2). The potential link between an increase in molting and the presence of PS-NH_2 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-NH_2 exposure will help to define how cationic nanoplastics may affect brine shrimp *A. franciscana* physiology and consequently survival and reproduction.

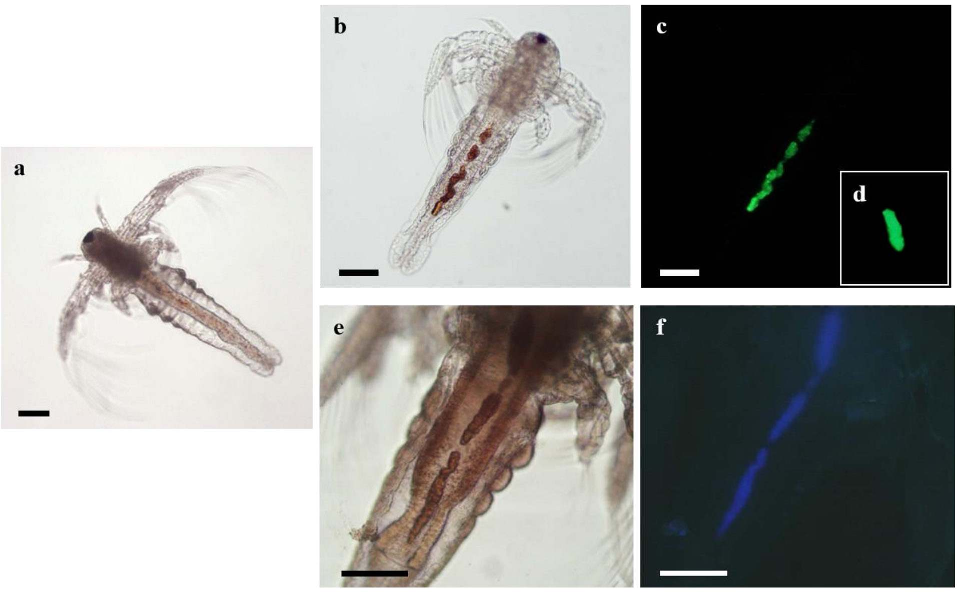

Figure 1. Effects of 40 nm PS-COOH and 50 nm PS-NH_2 on brine shrimp *A. franciscana* nauplii at 48 h: (a) control in NSW; (b, c) accumulation of green fluorescent PS-COOH (25 µg/ml) and (e, f) blue fluorescent PS-NH_2 (25 µg/ml) inside the digestive trait; (d) detail of fecal pellet containing PS-COOH aggregates. Images are representative of three independent experiments. Scale bar: 100 µm.

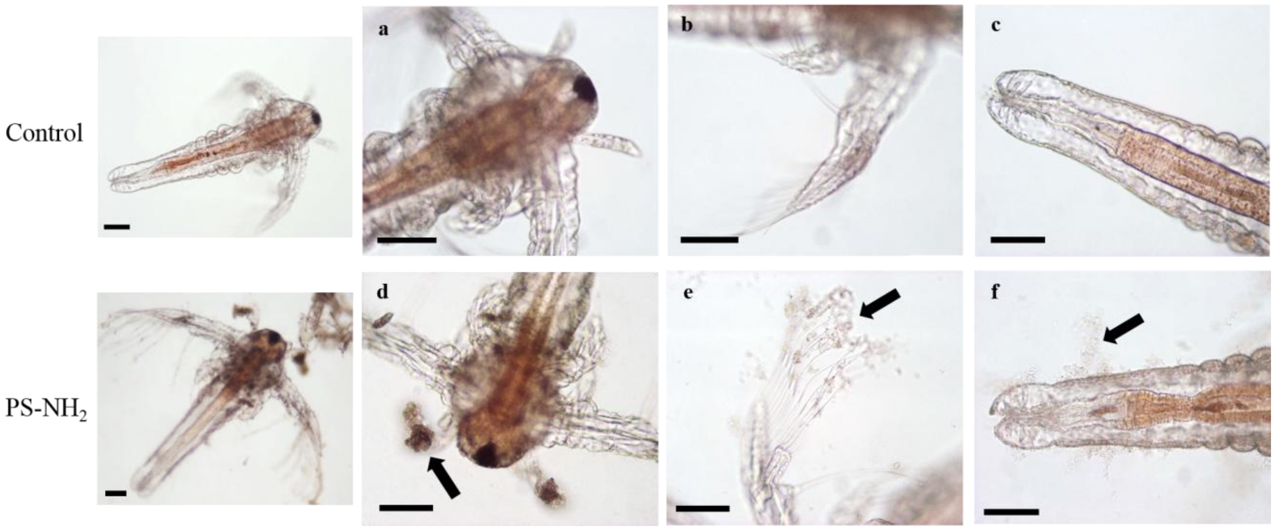

Figure 2. Effects of 50 nm PS-NH_2 on brine shrimp *A. franciscana* nauplii at 48 h. Upper images showing control in NSW: detail of clear (a) sensorial antennules, (b) antennae and (c) abdomen. Lower images showing PS-NH_2 (50 µg/ml) exposed nauplii (48 h): detail of nanoplastics attached to (d) sensorial antennules, (e) sensory hairs of the antennae and (f) abdomen region. Aggregates of PS-NH_2 are indicated by black arrows. Images are representative of three independent experiments. Scale bar: 100 µm.

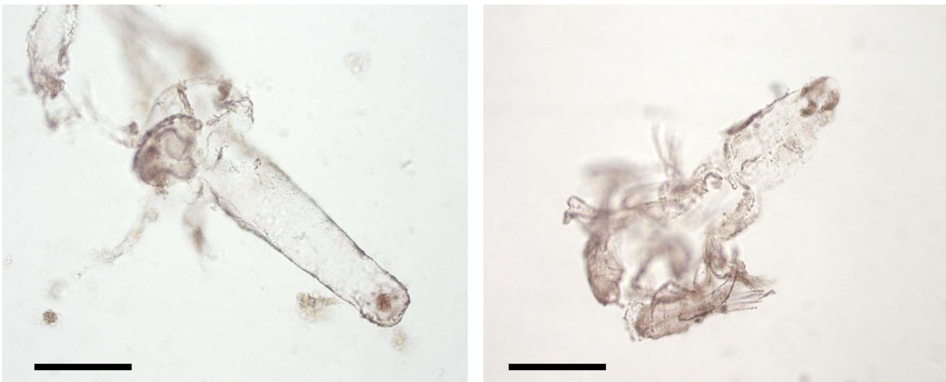

Figure 3. Two examples of molts found in wells containing brine shrimp *A. franciscana* nauplii after 48 h of exposure to 50 nm PS-NH_2. Anostracans such as *A. franciscana* are characterized by absence of carapace, therefore molts appeared quite transparent and easily broken by other brine shrimps swimming through. 10X images are referred to 50 µg/ml exposed organisms and representative of three independent experiments. Scale bar: 200 µm.

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 experiments) earlier exposed to PS-COOH (0, 5, 25, 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 trait of brine shrimp *A. franciscana*, even if a significant proportion was be retained in the gut (Ates 2013a, Ates 2013b). On the opposite, larvae exposed to PS-NH_2 (0, 5, 25, 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*.

4. 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 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.

Acknowledgements

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”.

PS NPs characterization was performed at the Centre for BioNano Interactions (University College of Dublin) funded by the Erasmus Long Life Learning Program (year 2012-2013) and based onworks supported by the EU FP7 Capacities project QualityNano (grant no. INFRA-2010-262163). We thank Claudia Faleri (Dept Life Sciences, University of Siena) for her assistance in TEM images.

References

Andrady, A. L., 2003. Plastics and the Environment. Wiley.

Arulvasu, C., Jennifer, S. M., Prabhu, D., Chandhirasekar, D., 2014. Toxicity effect of silver nanoparticles in brine shrimp Artemia. ScientificWorldJournal. 2014**,** 256919.

Ates, M., Daniels, J., Arslan, Z., Farah, I. O., 2013a. Effects of aqueous suspensions of titanium dioxide nanoparticles on Artemia salina: assessment of nanoparticle aggregation, accumulation, and toxicity. Environ Monit Assess. 185**,** 3339-48.

Ates, M., Daniels, J., Arslan, Z., Farah, I. O., Rivera, H. F., 2013b. Comparative evaluation of impact of Zn and ZnO nanoparticles on brine shrimp (Artemia salina) larvae: effects of particle size and solubility on toxicity. Environ Sci Process Impacts. 15**,** 225-33.

Auffan, M., Bertin, D., Chaurand, P., Pailles, C., Dominici, C., Rose, J., Bottero, J. Y., Thiery, A., 2013. Role of molting on the biodistribution of CeO2 nanoparticles within Daphnia pulex. Water Res. 47**,** 3921-30.

Bandyopadhyay, A., Basak, G. C., 2007. Studies on photocatalytic degradation of polystyrene. Material Science and Technology. 23**,** 307-317.

Barahona, M. V., Sánchez-Fortún, S., 1996. Comparative Sensitivity of Three Age Classes of *Artemia salina* Larvae to Several Phenolic Compounds. Bull. Environ. Contam. Toxicol. 56**,** 271-278.

Besseling, E., Wang, B., Lurling, M., Koelmans, A. A., 2014. Nanoplastic affects growth of S. obliquus and reproduction of D. magna. Environ Sci Technol. 48**,** 12336-43.

Bexiga, M. G., Kelly, C., Dawson, K. A., Simpson, J. C., 2014. RNAi-mediated inhibition of apoptosis fails to prevent cationic nanoparticle-induced cell death in cultured cells. Nanomedicine (Lond). 9**,** 1651-64.

Bexiga, M. G., Varela, J. A., Wang, F., Fenaroli, F., Salvati, A., Lynch, I., Simpson, J. C., Dawson, K. A., 2011. Cationic nanoparticles induce caspase 3-, 7- and 9-mediated cytotoxicity in a human astrocytoma cell line. Nanotoxicology. 5**,** 557-67.

Bramini, M., Ye, D., Hallerbach, A., Raghnaill, M. N., Salvati, A., A˚berg, C., Dawson, K. A., 2014. Imaging Approach to Mechanistic Study of Nanoparticle Interactions with the Blood-Brain Barrier. ACS Nano. 8**,** 4304–4312.

Browne, M. A., Crump, P., Niven, S. J., Teuten, E., Tonkin, A., Galloway, T., Thompson, R., 2011. Accumulation of microplastic on shorelines woldwide: sources and sinks. Environ Sci Technol. 45**,** 9175-9.

Canesi, L., Frenzilli, G., Balbi, T., Bernardeschi, M., Ciacci, C., Corsolini, S., Della Torre, C., Fabbri, R., Faleri, C., Focardi, S., Guidi, P., Kocan, A., Marcomini, A., Mariottini, M., Nigro, M., Pozo-Gallardo, K., Rocco, L., Scarcelli, V., Smerilli, A., Corsi, I., 2014. Interactive effects of n-TiO2 and 2,3,7,8-TCDD on the marine bivalve Mytilus galloprovincialis. Aquat Toxicol. 153**,** 53-65.

Casado, M. P., Macken, A., Byrne, H. J., 2013. Ecotoxicological assessment of silica and polystyrene nanoparticles assessed by a multitrophic test battery. Environ Int. 51**,** 97-105.

Chua, E. M., Shimeta, J., Nugegoda, D., Morrison, P. D., Clarke, B. O., 2014. Assimilation of polybrominated diphenyl ethers from microplastics by the marine amphipod, Allorchestes compressa. Environ Sci Technol. 48**,** 8127-34.

CNR, A. I., Metodo 8060 di valutazione della tossicità acuta con *Artemia* sp., Manuali e Linee Guida - Metodi analitici per le acque, Vol. 3. APAT IRSA CNR, 2003, pp. 1043-1049.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., Galloway, T. S., 2013. Microplastic ingestion by zooplankton. Environ Sci Technol. 47**,** 6646-55.

Cole, M., Webb, H., Lindeque, P. K., Fileman, E. S., Halsband, C., Galloway, T. S., 2014. Isolation of microplastics in biota-rich seawater samples and marine organisms. Sci Rep. 4**,** 4528.

Corsi, I., Cherr, G. N., Lenihan, H. S., Labille, J., Hassellov, M., Canesi, L., Dondero, F., Frenzilli, G., Hristozov, D., Puntes, V., Della Torre, C., Pinsino, A., Libralato, G., Marcomini, A., Sabbioni, E., Matranga, V., 2014. Common Strategies and Technologies for the Ecosafety Assessment and Design of Nanomaterials Entering the Marine Environment. Nano Focus. 8**,** 9694–9709.

Cozar, A., Echevarria, F., Gonzalez-Gordillo, J. I., Irigoien, X., Ubeda, B., Hernandez-Leon, S., Palma, A. T., Navarro, S., Garcia-de-Lomas, J., Ruiz, A., Fernandez-de-Puelles, M. L., Duarte, C. M., 2014. Plastic debris in the open ocean. Proc Natl Acad Sci U S A. 111**,** 10239-44.

Cozar, A., Sanz-Martin, M., Marti, E., Gonzalez-Gordillo, J. I., Ubeda, B., Galvez, J. A., Irigoien, X., Duarte, C. M., 2015. Plastic accumulation in the mediterranean sea. PLoS One. 10**,** e0121762.

Della Torre, C., Balbi, T., Grassi, G., Frenzilli, G., Bernardeschi, M., Smerilli, A., Guidi, P., Canesi, L., Nigro, M., Monaci, F., Scarcelli, V., Rocco, L., Focardi, S. E., Monopoli, M., Corsi, I., 2015. Titanium dioxide nanoparticles modulate the toxicological response to cadmium in the gills of Mytilus galloprovincialis. J Hazard Mater. 297, 92-100.

Della Torre, C., Bergami, E., Salvati, A., Faleri, C., Cirino, P., Dawson, K. A., Corsi, I., 2014a. Accumulation and embryotoxicity of polystyrene nanoparticles at early stage of development of sea urchin embryos Paracentrotus lividus. Environ Sci Technol. 48**,** 12302-11.

Della Torre, C., Bocci, E., Focardi, S. E., Corsi, I., 2014b. Differential ABCB and ABCC gene expression and efflux activities in gills and hemocytes of Mytilus galloprovincialis and their involvement in cadmium response. Mar Environ Res. 93**,** 56-63.

EPA, U., Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. 2002.

Eriksen, M., Lebreton, L. C., Carson, H. S., Thiel, M., Moore, C. J., Borerro, J. C., Galgani, F., Ryan, P. G., Reisser, J., 2014. Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. PLoS One. 9**,** e111913.

Farrell, P., Nelson, K., 2013. Trophic level transfer of microplastic: Mytilus edulis (L.) to Carcinus maenas (L.). Environ Pollut. 177**,** 1-3.

Galgani, F., Fleet, D., Van Franeker, J., Katsanevakis, S., Maes, T.,, Mouat, J., Oosterbaan, L., Poitou, I., Hanke, G., Thompson, R., Amato, E.,, Birkun, A., Janssen, C., Marine Strategy Framework Directive Task Group 10 Report Marine Litter. In: N. Zampoukas, (Ed.), 2010, pp. 57.

Gambardella, C., Mesaric, T., Milivojevic, T., Sepcic, K., Gallus, L., Carbone, S., Ferrando, S., Faimali, M., 2014. Effects of selected metal oxide nanoparticles on Artemia salina larvae: evaluation of mortality and behavioural and biochemical responses. Environ Monit Assess. 186**,** 4249-59.

Garaventa, F., Gambardella, C., Di Fino, A., Pittore, M., Faimali, M., 2010. Swimming speed alteration of Artemia sp. and Brachionus plicatilis as a sub-lethal behavioural end-point for ecotoxicological surveys. Ecotoxicology. 19**,** 512-9.

Handy, R. D., Owen, R., Valsami-Jones, E., 2008. The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs. Ecotoxicology. 17**,** 315-25.

Hofer, T. N., 2008. Marine Pollution: New Research. Nova Science Publishers.

Innocenti, F. D., Biodegradability and compostability. In: E. Chiellini, Solaro, R., (Ed.), Biodegradable Polymers and Plastics. Kluwer Academic/Plenum Publishers, New York, 2003, pp. 33-46.

Klaine, S. J., Alvarez, P. J., Batley, G. E., Fernandes, T. F., Handy, R. D., Lyon, D. Y., Mahendra, S., McLaughlin, M. J., Lead, J. R., 2008. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. Environ Toxicol Chem. 27**,** 1825-1851.

Lee, K. W., Shim, W. J., Kwon, O. Y., Kang, J. H., 2013. Size-dependent effects of micro polystyrene particles in the marine copepod Tigriopus japonicus. Environ Sci Technol. 47**,** 11278-83.

Lesniak, A., Campbell, A., Monopoli, M. P., Lynch, I., Salvati, A., Dawson, K. A., 2010. Serum heat inactivation affects protein corona composition and nanoparticle uptake. Biomaterials. 31**,** 9511-8.

Lithner, D., Larsson, A., Dave, G., 2011. Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. Sci Total Environ. 409**,** 3309-24.

Liu, Y., Li, W., Lao, F., Liu, Y., Wang, L., Bai, R., Zhao, Y., Chen, C., 2011. Intracellular dynamics of cationic and anionic polystyrene nanoparticles without direct interaction with mitotic spindle and chromosomes. Biomaterials. 32**,** 8291-303.

Loos, C., Syrovets, T., Musyanovych, A., Mailander, V., Landfester, K., Nienhaus, G. U., Simmet, T., 2014. Functionalized polystyrene nanoparticles as a platform for studying bio-nano interactions. Beilstein J Nanotechnol. 5**,** 2403-12.

Lunov, O., Syrovets, T., Loos, C., Beil, J., Delacher, M., Tron, K., Nienhaus, G.U., Musyanovych, A., Mailander, V., Landfester, K., Simmet, T., 2011. Differential Uptake of Functionalized Polystyrene Nanoparticles by Human Macrophages and a Monocytic Cell Line. ACS Nano. 5**,** 1657–1669.

Makridis, P., Vadstein, O., 1999. Food size selectivity of Artemia franciscana at three developmental stages. Journal of Plankton Research. 21**,** 2191–2201.

Manzo, S., Miglietta, M. L., Rametta, G., Buono, S., Di Francia, G., 2013. Embryotoxicity and spermiotoxicity of nano-sized ZnO for Mediterranean sea urchin Paracentrotus lividus. J Hazard Mater. 254-255**,** 1-9.

Matranga, V., Corsi, I., 2012. Toxic effects of engineered nanoparticles in the marine environment: model organisms and molecular approaches. Mar Environ Res. 76**,** 32-40.

Moore, M. N., 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? Environ Int. 32**,** 967-76.

Murray, F., Cowie, P. R., 2011. Plastic contamination in the decapod crustacean Nephrops norvegicus (Linnaeus, 1758). Mar Pollut Bull. 62**,** 1207-17.

Nowack, B., Bucheli, T. D., 2007. Occurrence, behavior and effects of nanoparticles in the environment. Environ Pollut. 150**,** 5-22.

Nunes, B. S., Carvalho, F. D., Guilhermino, L. M., Van Stappen, G., 2006. Use of the genus Artemia in ecotoxicity testing. Environ Pollut. 144**,** 453-62.

Persoone, G., Wells, P. G., *Artemia* in aquatic toxicology: a review. In: P. Sorgeloos, Bengtson, D. A., Decleir, W., Jaspers, E., (Ed.), *Artemia* research and its applications. Universa Press, Wetteren, Belgium, 1987, pp. 380.

Pretti, C., Oliva, M., Pietro, R. D., Monni, G., Cevasco, G., Chiellini, F., Pomelli, C., Chiappe, C., 2014. Ecotoxicity of pristine graphene to marine organisms. Ecotoxicol Environ Saf. 101**,** 138-45.

Rochman, C. M., Manzano, C., Hentschel, B. T., Simonich, S. L., Hoh, E., 2013. Polystyrene plastic: a source and sink for polycyclic aromatic hydrocarbons in the marine environment. Environ Sci Technol. 47**,** 13976-84.

Rodd, A. L., Creighton, M. A., Vaslet, C. A., Rangel-Mendez, J. R., Hurt, R. H., Kane, A. B., 2014. Effects of surface-engineered nanoparticle-based dispersants for marine oil spills on the model organism Artemia franciscana. Environ Sci Technol. 48**,** 6419-27.

Rossi, G., Barnoud, J., Monticelli, L., 2014. Polystyrene Nanoparticles Perturb Lipid Membranes. The Journal of Physical Chemistry Letters. 5**,** 241-246.

Ruppert, E. E., Fox, R. S., Barnes, R. D., 2004. Invertebrate Zoology: A Functional Evolutionary Approach. Thomson-Brooks/Cole.

Salvati, A., Åberg, C., dos Santos, T., Varela, J., Pinto, P., Lynch, I., Dawson, K. A., 2011. Experimental and theoretical comparison of intracellular import of polymeric nanoparticles and small molecules: toward models of uptake kinetics. Nanomedicine. 7**,** 818-26.

Setala, O., Fleming-Lehtinen, V., Lehtiniemi, M., 2014. Ingestion and transfer of microplastics in the planktonic food web. Environ Pollut. 185**,** 77-83.

Silvestre, C., Duraccio, D., Cimmino, S., 2011. Food packaging based on polymer nanomaterials. Progress in Polymer Science. 36**,** 1766-1782.

SIRA RSS. www. sira.arpat.toscana.it

Snell, T. W., Hicks, D. G., 2011. Assessing toxicity of nanoparticles using Brachionus manjavacas (Rotifera). Environ Toxicol. 26**,** 146-52.

Stone, V., Nowack, B., Baun, A., van den Brink, N., Kammer, F., Dusinska, M., Handy, R., Hankin, S., Hassellov, M., Joner, E., Fernandes, T. F., 2010. Nanomaterials for environmental studies: classification, reference material issues, and strategies for physico-chemical characterisation. Sci Total Environ. 408**,** 1745-54.

UNEP, Marine Debris as a Global Environmental Problem. UNEP Scientific and Technical Advisory Panel, 2011, pp. 40.

Vanhaecke, P., Persoone, G., Report on an Intercalibration exercise on a Short-term Standard Toxicity Test with Artemia nauplii (ARC-TEST). Vol. 106. Artemi a Reference Center, Laboratory for Biological Research in Aquatic Pollution and Laboratory for Mari cu!ture, Ghent, Belgium, 1981, pp. 359-376.

Velzeboer, I., Kwadijk, C. J., Koelmans, A. A., 2014. Strong sorption of PCBs to nanoplastics, microplastics, carbon nanotubes, and fullerenes. Environ Sci Technol. 48**,** 4869-76.

Wang, F., Bexiga, M. G., Anguissola, S., Boya, P., Simpson, J. C., Salvati, A., Dawson, K. A., 2013a. Time resolved study of cell death mechanisms induced by amine-modified polystyrene nanoparticles. Nanoscale. 5**,** 10868-76.

Wang, F., Yu, L., Monopoli, M. P., Sandin, P., Mahon, E., Salvati, A., Dawson, K. A., 2013b. The biomolecular corona is retained during nanoparticle uptake and protects the cells from the damage induced by cationic nanoparticles until degraded in the lysosomes. Nanomedicine. 9**,** 1159-68.

Ward, J. E., Kach, D. J., 2009. Marine aggregates facilitate ingestion of nanoparticles by suspension-feeding bivalves. Mar Environ Res. 68**,** 137-42.

Watts, A. J., Lewis, C., Goodhead, R. M., Beckett, S. J., Moger, J., Tyler, C. R., Galloway, T. S., 2014. Uptake and retention of microplastics by the shore crab Carcinus maenas. Environ Sci Technol. 48**,** 8823-30.

Wegner, A., Besseling, E., Foekema, E. M., Kamermans, P., Koelmans, A. A., 2012. Effects of nanopolystyrene on the feeding behavior of the blue mussel (Mytilus edulis L.). Environ Toxicol Chem. 31**,** 2490-7.

Wright, S. L., Rowe, D., Thompson, R. C., Galloway, T. S., 2013a. Microplastic ingestion decreases energy reserves in marine worms. Curr Biol. 23**,** R1031-3.

Wright, S. L., Thompson, R. C., Galloway, T. S., 2013b. The physical impacts of microplastics on marine organisms: a review. Environ Pollut. 178**,** 483-92.

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

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Supplementary material

This document is an electronic supplement which provides additional information about this work.

It is organized as follows:

Figure S1

Figure S2

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A B

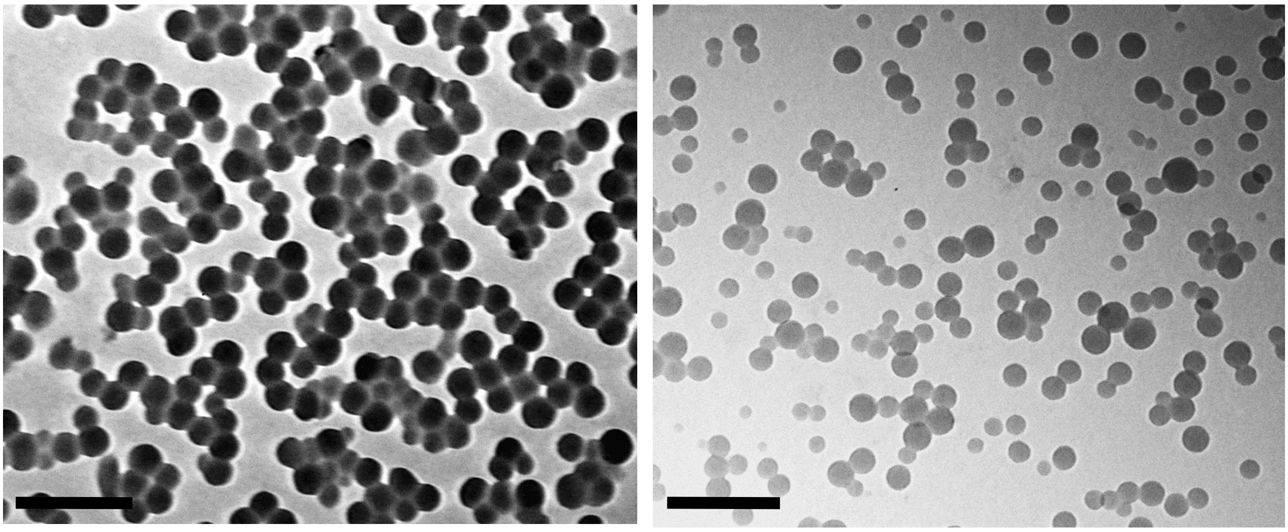

Figure S1. Primary characterization of PS NPs: TEM images showing an average size value of 42 ± 1.9 nm PS-COOH (A) and 53 ± 2 nm PS-NH_2 (B) suspended in distilled water. Scale bar: 200 nm.

Figure S2. Still of video data showing brine shrimp *A. salina larvae* after 48 h of exposure to PS-COOH (25 µg/ml), excreting a pellet containing PS-COOH aggregates. Scale bar: 100 µm.

Video

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Video Still

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