Long-term toxicity of surface-charged polystyrene nanoplastics to marine planktonic species Dunaliella tertiolecta and Artemia franciscana

Long-term toxicity of surface charged polystyrene nanoplastics to marine planktonic species *Dunaliella tertiolecta* and *Artemia franciscana*

Bergami E.[1*], Pugnalini S.[1], Vannuccini M. L.[1], Manfra L.[2, 3], Faleri C.[4], Savorelli F.[5], Dawson K. A.[56], Corsi I.[1]

[1]Department of Physical, Earth and Environmental Sciences, University of Siena, Italy;

[2]Institute for Environmental Protection and Research (ISPRA), Rome, Italy;

[3]Department of Biology and Evolution of Marine Organisms, Anton Dohrn Zoological Station, 80121 Naples, Italy;

[4]Department of Life Sciences, University of Siena, 53100 Siena, Italy;

[5]Regional Agency for Prevention, Environment and Energy of Emilia-Romagna (Arpae ER), Ferrara, Italy

[6]Centre for BioNano Interactions, School of Chemistry and Chemical Biology, University College Dublin, Ireland;

***Corresponding author:** Elisa Bergami; bergami@student.unisi.it; Department of Physical, Earth and Environmental Sciences, University of Siena, Italy.

Abstract

Plastic pollution has been globally recognized as a critical issue for marine ecosystems and nanoplastics constitute one the last ~~frontier~~ unexplored areas to understand the magnitude of this threat. However, current difficulties in sampling and identifying nano-sized debris make hard to assess their occurrence in marine environment. Polystyrene nanoparticles (PS NPs) ~~have~~ are~~been recently adopted~~ largely used as ~~model for~~ nanoplastics in ecotoxicological studies and ~~despite~~ although acute exposures have been already investigated, long-term toxicity on marine organisms is lacking. Our study aims at evaluating the effects of 40 nm PS anionic carboxylated (PS-COOH) and 50 nm cationic amino-modified (PS-NH_2) NPs in two planktonic species, the green microalga *Dunaliella tertiolecta* and the brine shrimp *Artemia franciscana*, respectively prey and predator. PS NP behaviour in exposure media was determined through DLS, while their toxicity to microalgae and brine shrimps evaluated through 72 h growth inhibition test and 14 d~~ays~~ long-term toxicity test respectively. Moreover, the expression of target genes (i.e. *clap* and *cstb*), having a role in brine shrimp larval growth and molting, was measured in 48 h brine shrimp larvae. A d~~D~~ifferent behaviour ~~-~~of the two PS NPs in exposure media as well as diverse toxicity ~~was observed to~~in the two planktonic species~~ – was observedupon PS NP exposure~~. PS-COOH formed micro-scale aggregates~~objects~~ (Z-Average > 1 µm) and did not affect the growth of microalgae up to 50 µg/ml as well as brine shrimps up to 10 µg/ml. However, these negatively charged NPs were adsorbed on microalgae and accumulated (and excreted) in brine shrimps, suggesting a potential trophic transfer from prey to predator. On the opposite, PS-NH_2 formed nano-scale aggregates (Z-Average < 200 nm)~~, and~~ caused inhibition of algal growth (EC_{50} = 12.97 µg/ml) and mortality in brine shrimps at 14 d (LC_{50} = 0.83 µg/ml). Moreover, 1 µg/ml PS-NH_2 ~~(at 1 µg/ml)~~ significantly induced *clap* and *cstb* genes, explaining the physiological alterations (e.g. increase in molting) previously observed in 48 h larvae, but also suggesting an apoptotic pathway triggered by cathepsin L-like protease in brine shrimps upon PS-NH_2 exposure. These findings provide a first insight into long-term toxicity of

nanoplastics to marine plankton, underlining the role of the surface chemistry in determining the behaviour and effects of PS NPs, in terms of adsorption, growth inhibition, accumulation, gene modulation and mortality. The use of long-term end-point has been identified as ~~more~~ valuable tool for assessing the impact of nanoplastics on marine planktonic species, being more predictable of real exposure scenarios for risk assessment purposes.

Keywords: ~~nanoplastics,~~ surface charge~~d~~, polystyrene nanoparticles, ecotoxicity, marine plankton, growth inhibition~~*Artemia franciscana*~~, ~~*Dunaliella*~~ cathepsin L-like protease~~tertiolecta~~.

1. Introduction

Plastic debris have been globally recognized as a menace for marine ecosystems (Andrady, 2011), being the major portion (between 60 and 80% and up to 96.87%) of the marine litter found during monitoring surveys (Gregory and Ryan, 1997; Ruiz-Orejón et al., 2016).

The evaluation of the impacts of the smallest ~~*invisible*~~ fraction of plastic debris, defined as nanoplastics (< 1 µm) (Hartmann et al., 2015), constitutes one of the last unexplored areas~~the last frontier in order~~ to fully understand the importance of this emerging threat for the marine environment.

The amount of nanoplastics spread in the oceans is currently unexplored, since ~~convectional~~ conventional sampling methods (i.e. neuston nets having mesh size > 300 µm) as well as analytical techniques available for the identification of plastic polymers still prevent to isolate and quantify the nano-fraction (Cózar et al., 2014; Koelmans et al., 2015).

Notwithstanding, Zhang and co-authors (2012) were able to measure polystyrene nanoparticles (PS NPs) in the range of 22 – 220 nm~~,~~ from the thermal cutting of polystyrene foam and later Lambert and Wagner (2016) found PS NPs in water suspensions obtained from weathered bulk polystyrene material under controlled laboratory conditions. This latter study demonstrated the occurrence of nanofragmentation of plastic debris in the aquatic environment as a consequence of several processes: UV-radiation, thermo-oxidation, hydrolysis, mechanical abrasion and not least biological degradation (Andrady, 2011). Such weathering processes may confer to the nano-sized fragments ~~new peculiar~~novel properties (Andrady, 2017), as for engineered nanomaterials (Klaine et al., 2012). For example, weathered plastic debris can be characterised by an increase in crystallinity due to oxidation (Rouillon et al., 2016) and acquire carbonyl functionalities as well as negative surface charges (Fotopoulou and Karapanagioti, 2012).

Nano-sized plastics can ~~thus~~ be easily ingested by organisms (Cole et al., 2013), pass biological barriers (Moore, 2006), penetrate tissues (Kashiwada, 2006) and even bioaccumulate in organs and tissues (von Moos et al., 2012). ~~Moreover~~T, ~~t~~heir high surface area can lead to exceptionally strong sorption of toxic compounds (e.g. PCBs, PAH and DDTs) (Rios et al., 2007; Rochman et al., 2013; Velzeboer et al., 2014), with potential added chemical toxicity, once NPs have passed the cell membranes (Bexiga et al., 2011; Salvati et al., 2011). Any adverse effect of nanoplastics will be thus related to their nano-scale properties, to polymer-associated chemicals (i.e. additives) or both, and will mainly depend on particle size, polymer type and aging (Besseling et al., 2014). Therefore, it is necessary to understand how nanoplastics behave in the marine environment depending on the chemical nature of the polymer and its surface modifications. Since the marine environment could be seen as the last reservoir of plastic debris, phyto- and zooplanktonic organisms can be seriously affected by the presence ~~are among the primary biological targets~~ of nanoplastics, being exposed to a variety of low-density polymeric ~~beads~~ particles along the ~~whole~~ water column (Manzo et al., 2013; Matranga and Corsi, 2012; Moore, 2006).

In order to evaluate the impact of nanoplastics on the wildlife, polymeric nano-scale particles, such as PS NPs having a polystyrene core and variable surface functional groups, can be used. These NPs are synthesized for a wide range of applications (Nowak and Bucheli, 2007), including biosensors (Velev and Kaler, 1999), photonics (Rogach et al., 2000), nanocomposites (Merinska and Dujkova, 2012) and drug delivery tools (Popielarski et al., 2005; Yap and Zhang, 2007). Common PS NPs include anionic carboxylated (-COOH) and cationic amino ($-NH_2$) surface modifications (Loos et al., 2014), which allow them to pass more easily through the cell membrane, as they share a similar molecular structure to proteins (Rossi et al., 2014). Several studies on cell lines confirmed their cellular uptake (Johnston et al., 2010; Salvati et al., 2011; Wang et al., 2012; Loos et al., 2014) as well as toxicity (Bexiga et al., 2011; Frölich et al., 2012). In particular, positive PS-NH_2 NPs were shown to trigger specific *in vitro* toxicity mechanisms ~~*in vitro*~~ including

lysosomal damage and ROS generation followed by apoptotic pathways through the induction of cathepsins and caspases (Wang et al., 2013).

Ecotoxicity studies about nanoplastics impact on aquatic organisms have exponentially increased in the last seven years and the majority adopted both PS-COOH and PS-NH_2 as model NPs (Bhattacharya et al., 2010, Wegner et al., 2012; Casado et al., 2013; Besseling et al., 2014, Della Torre et al., 2014, Cole and Galloway, 2015; Bergami et al., 2016; Canesi et al., 2015, Canesi et al., 2016; Pinsino et al., 2017).

Our previous findings on zooplankton (Della Torre et al., 2014; Bergami et al., 2016; Pinsino et al., 2017) showed diverse effects on sea urchin embryonic development (*Paracentrotus lividus*) and brine shrimp larval growth (*Artemia franciscana*) depending on the surface charge of PS NPs. Indeed, potential toxicity associated to the PS core could be neglected, since it does not degrade under environmental conditions even in long-term studies (Besseling et al., 2014; Loos et al., 2014).

After short-term exposures (48 h), negatively charged ~~negative~~ PS-COOH aggregates were strongly retained in the digestive tract of sea urchin embryos and brine shrimp larvae, while positively charged NPs seriously affected the development and growth of these species. In sea urchin embryos, PS-NH_2 were able to elicit developmental defects through the induction of genes related to stress (i.e. *hsp70*) but also apoptosis (*cas8*), similarly to previous findings in human cell lines (Wang et al., 2013).

In brine shrimp larvae, PS-NH_2 were mostly stuck to the external appendices, clearly impairing their swimming and increasing the molting of about 50% respect to the control (Bergami et al., 2016). However, whereas EC_{50} values were determined for sea urchin embryos exposed to PS-NH_2 (Della Torre et al., 2014), for brine shrimp larvae it was not possible to discriminate the toxicity of PS NPs through the standard acute test (Artoxkit, 2014) and the mechanism behind the physiological alterations observed was not fully elucidated.

In ecotoxicology, the acute toxicity is determined at first and mainly in terms of mortality, with model organisms exposed to high concentrations of contaminants over a short time period. However, any result based only on short-term and high exposure concentrations hamper extrapolation of data to a more realistic scenario (Rand, 1995). On the contrary, long-term toxicity tests follow as needed, usually lasting for 10% of the organism's lifespan (Newman, 2010) and focus on both mortality and sub-lethal effects (e.g. growth and reproduction) (Cattaneo et al., 2009). Such potential alterations, as a result of a long-term exposure, constitute a ~~the ultimate~~more reliable endpoint (Rand, 1995) and represent the most appropriate tool for studying emerging contaminants (Comfort et al., 2014) including nanoplastics. For example, Besseling and co-authors (2014) observed reduced body size and alteration in the reproduction of *Daphnia magna* exposed for 21 days to 70 nm PS-COOH.

Considering the brine shrimp of the genus *Artemia* as model marine organism~~s~~, Rotini et al. (2015) recently compared the EC_{50} values obtained from different ~~ecotoxicity tests~~assays, showing the reliability of the long-term toxicity test compared to hatching and acute tests.

Our study aims at evaluating the effects caused by two different surface charged nanoplastics (40 nm ~~PS anionic carboxylated (~~PS-COOH~~)~~ and 50 nm ~~cationic amino-modified (~~PS-NH_2) ~~NPs~~ on two planktonic species, the green microalga *Dunaliella tertiolecta* and the brine shrimp *Artemia franciscana*, through 72 h growth inhibition test and 14 d long-term toxicity test respectively. PS NP disposition as well as their consequences to marine plankton (i.e. surface adsorption, accumulation, growth inhibition and mortality) were assessed. Moreover, the expression of *clap* and *cstb* genes related to larval growth and molting was determined in 48 h brine shrimp larvae exposed to PS-NH_2.

These model organisms represent the first levels at the bottom of the marine trophic web, respectively as prey and predator, thus any negative effect on them leads to serious repercussions on the health of the marine ecosystem. Both negatively (-COOH) and positively (-NH_2) surface

charged PS NPs were used in order to correlate the different functionalization (i.e. surface chemistry) to the observed toxicity.

2. Materials and Methods

2.1. PS NP behaviour in exposure media

40 nm yellow-green fluorescently labelled PS-COOH (505 nm excitation, 515 nm emission) were purchased from Invitrogen, whereas 50 nm unlabelled PS-NH_2 NPs from Bangs Laboratories Inc. Functionalized PS NPs (anionic PS-COOH and cationic PS-NH_2) are the most common nanoplastics used in ecotoxicological studies on aquatic organisms published in the last 7 years (Bhattacharya et al., 2010, Besseling et al., 2014, Della Torre et al., 2014, Cole and Galloway, 2015, Canesi et al., 2015, Canesi et al., 2016, Pinsino et al., 2017). In this study, the use of the same batches for PS NPs allowed us to compare the obtained results with our previous findings on brine shrimp ~~as model organism~~ (Bergami et al., 2016).

PS NP stock solutions were supplied in deionised ~~milli-Q~~ water without any surfactants or preservatives ~~(mQW) used for ecotoxicity tests~~and contained 50 and 100 mg/ml of PS-COOH and PS-NH_2 respectively. Concerning the fluorescent labelling of PS-COOH, the dye was contained inside the polymer matrix instead of being attached on the surface. Therefore, any potential toxicity related to the dye was considered negligible.

Primary characterization of PS NPs was performed as reported in Bergami et al. (2016). Secondary characterization of PS NPs in milli-Q water (mQW), microalgae medium (prepared in 0.45 µm filtered NSW, T = 20°C, salinity 30‰, pH 8.3) and natural sea water (NSW) (0.45 µm filtered, T = 25°C, salinity 38‰, pH 8.3) was made using Dynamic Light Scattering (DLS, Malvern instruments), combined with the Zetasizer Nano Series software, version 7.02 (Particular Sciences,

UK). Z-average (nm), Polydispersity Index (PDI, dimensionless) and Zeta (ζ-) potential (mV) were measured as key parameters describing NP behaviour in complex environmental media (SCENIHR, 2007; Stone et al., 2010). Measurements were carried out in triplicate, each containing 11 runs of 10 second for size parameters, 20 runs for ζ-potential.

Ecotoxicity tests were performed in NSW-based media collected from the Tuscany archipelago area (Tyrrhenian Sea) (Bergami et al., 2016). Physico-chemical parameters of NSW included: TOC 1.3%, total oxygen 6.6 mg/l, total PAH 0.12 mg/Kg, Cr < 1 µg/l, As 1 µg/l, Cd 0.09 µg/l, Hg 0.02 µg/l, Pb < 1 µg/l (data available at: SIRA RSS www.sira.arpat.toscana.it/). PS NP final suspensions in NSW were prepared from the stock solutions and quickly vortexed without sonication prior to use, since no important changes in PS NP size measurements in NSW with and without sonication have been previously observed (see Della Torre et al., 2014 SI). This method of dispersion can be seen as a more realistic representation of natural conditions occurring for nanoplastics in the marine environment (Bhattacharya et al., 2010).

PS NP concentrations used are reported as µg/ml (for particle numbers, refer to Table S1 in Supporting Information).

2.2. Ecotoxicity

In this study, the toxicity of PS nanoplastics was evaluated on two planktonic species, ~~as~~ the unicellular green microalga *D. tertiolecta*, supplied by the Regional Agency for Environmental Protection (ARPA Emilia-Romagna), and the microcrustacean brine shrimp *A. franciscana* ~~larvae~~, purchased from MicroBioTests (Ghent, Belgium). Growth inhibition test (72 h) and long-term lethal test (14 d) were conducted, respectively, on the green microalga and the microcrustacean by testing both PS NPs following standardized procedures, as described below. Microalgae were

further used to feed brine shrimp larvae during long-term lethal toxicity test~~, using a prey-to-predator approach~~.

2.2.1. Growth Inhibition of the green microalga

The growth inhibition test (72 h) using green microalgae was performed according to a standard guideline (ISO, 2006) with some modifications as follow. Sterile microalgae medium was prepared in NSW (salinity adjusted to 30‰) by the addition of f/2 Guillard and then autoclaved. Experiments were performed in 24-well plates, with average initial cell density of $7 \cdot 10^5$ cells/ml. Tested PS NPs were 0.5 - 1 - 5 - 10 - 25 - 50 µg/ml and microalgae medium was used as control. For each group, three replicates were run and each experiment repeated at least three times for each PS NP. During the test, microalgae were incubated under controlled static conditions (20±1°C, photoperiod of 16:8 h light:darkness, without shaking). After 72 h, microalgae were fixed in 1:1 lugol:ethanol solution and cell density estimated by counting under optical microscope Olympus BX51 (40X), equipped with a Neubauer Improved chamber. The number of cells/ml, growth rate (µ) and inhibition of growth rate ($I_{\mu i}$) were determined. EC_{50} were calculated by plotting the $I_{\mu i}$ against the logarithm of the concentration of PS NP tested.

2.2.2. Short- and long-term toxicity on the brine shrimp

In order to provide new insight into the expression to genes related to physiological alterations specifically provoked by PS-NH_2 in brine shrimp larvae after 48 h (Bergami et al., 2016), ~~Hatching of brine shrimp cysts and~~ short-term toxicity test ~~were~~ was performed ~~as previously described in Bergami et al. (2016)~~accordingly, with some modifications as reported. Briefly, about 200 newly hatched nauplii (Instar I nauplius stage) were exposed to PS-NH_2 suspensions (at 0, 0.1 and 1 µg/ml) prepared in NSW in glass beakers (final volume of 100 ml) and incubated at 25±1°C under~~in~~

dark static conditions, without providing food. At 48 h, all swimming larvae were collected and stored at -80°C for gene expression analysis. The experiment was performed in triplicates and repeated three times.

The l~~L~~ong-term toxicity of both PS NPs (PS-COOH and PS- NH_2) ~~test~~ was then carried out according to Savorelli et al. (2007) and Manfra et al. (2012), with some changes concerning the preparation of the newly hatched larvae obtained according the protocol available from Artoxkit (2014), since we decided to maintain the same conditions as Bergami et al. (2016), for hatching and start the long-term toxicity test using brine shrimp larvae at Instar I nauplius stage.

Preliminarily, PS NP concentrations tested for evaluating the long-term toxicity were 1 and 10 µg/ml. The former one~~,~~ correspond~~sing~~ to about 1.5 – 2.8 $\cdot 10^{10}$ NPs/ml (Table S1), ~~could be representative of a putative environmental concentration of nanoplastics in the marine environment (Lambert and Wagner, 2016),~~ while the second ~~concentration~~ one was in the same order of magnitude of the EC_{50} calculated for PS-NH_2 in the growth inhibition test (see paragraph 3.2. in the results section). However, due to the high mortality observed for brine shrimps exposed to PS-NH_2 in the first attempts, a further concentration series for this positively charged nanoplastic was set to 0.5 - 1 - 1.5 - 2.5 - 5 µg/ml.

PS NP test solutions were prepared in 50 ml flasks (final volume of 30 ml) containing NSW, to which a microalgae inoculum (to reach a final density of 10^5 cells/ml) and 10 newly hatched brine shrimp nauplii were added in sequence. For the whole duration of the test, the flasks were maintained under controlled conditions (25±1°C, photoperiod of 16:8 h light:darkness) and test solutions renewed every 2-3 days (at 2, 5, 7, 9 and 12 d). Three replicates were set for each PS NP concentration and the control group (containing only NSW and microalgae) and the test was repeated at least three times. As end-point, the number of dead larvae after 14 days of exposure was recorded on a worksheet and the percentage of mortality at each concentration calculated. Brine shrimps were considered dead if they did not display any movement for 10 seconds (Gambardella et

al., 2014). The test was considered acceptable if the control group displayed an average mortality ≤ 20 %. LC_{50} value and 95% confidence limits were calculated by comparison of the mortality at each concentration to the control group, using EPA Probit Analysis Program (version 1.5).

In order to further investigate the effect of PS-NH_2 on larval development, images of brine shrimps from the 14 days toxicity test were taken through optical microscopy (Olympus BX51) and analysed using ImageJ software (Version 1.49, Wayne Rasband, National Institutes of Health, USA). For each organism, the length from the cephalic region (i.e. nauplius eye) to the telson was measured. Antennule and caudal rami were not considered in the measurements, since these parts were usually present at different focus distances with respect to the main body (i.e. thorax and abdomen) of the animal.

2.3. Disposition of PS nanoplastics in planktonic organisms

Disposition of fluorescent PS-COOH (at 5 μg/ml) was assessed in 72 h exposed green microalgae, washed twice in NSW and immobilized on slides pre-treated with 0.05% protamine sulfate and nuclei labelled with DAPI. As negative control, microalgae ~~in~~ suspensions without NPs were also considered. Cells were observed under optical fluorescent microscope AXIO IMAGER Z1 using Apotome system (Zeiss), with filter FITC 470/525 for PS-COOH and filter DAPI 365/445 for nuclei staining. Images were taken with AxioCam MRm camera at 63X using Axio Vision Software.

Disposition of unlabelled PS-NH_2 (at 5 μg/ml) was determined on microalgae surface using scanning electron microscopy (SEM), according to the method described by Miller et al. (2012) with some modifications. Briefly, cells were centrifuged (20°C, 1600 rpm, 3') and pellets fixed in 5% glutaraldehyde for 1 h, dehydrated in a graded series of ethanol up to 70% and maintained at 4°C until further analysis. As negative control, microalgae ~~in~~ suspensions without NPs were

considered. For the observation at SEM, samples were placed on a glass coverslip coated with a thin layer of poly-l-lysine dehydrated in absolute ethanol and subsequently subjected to the critical point drying. At last, samples were metallized and observed under SEM (Philips XL20), operating at 100 KW accelerating voltage.

Concerning the brine shrimp *A. franciscana*, the disposition of fluorescent PS-COOH (at 10 μg/ml) was determined under optical fluorescent microscope (Olympus BX51) at 14 days of long-term toxicity tests.

2.4. RNA extraction, cDNA synthesis and Real Time q-PCR

Gene expression analysis was performed following the procedure reported in Vannuccini et al. (2015). RNA was extracted using reagent RNA extraction buffer (Sigma, UK) in 2 mL centrifuge tubes using a Tissue Lyser (Qiagen, Hilden, Germany) and precipitated by adding 0.5 vol. of isopropyl alcohol and 0.5 vol. of RNA precipitation solution (1.2 M sodium chloride, 0.8 M disodium citrate). Three independent RNA isolations were performed for each sample. RNA concentrations were measured using a traycell spectrophotometer (Eppendorf) at 260 nm λ and RNA quality confirmed on 1% agarose gel, showing discrete 18 S and 28 S ribosomal RNA bands. Total RNA (200 ng) was transcribed to cDNA using qScript™ XLT One-Step RT-qPCR ToughMix® (Quanta Biosciences) according to manufacturer's protocol.

The expression of selected genes related to molting in brine shrimp larvae, as *cathepsin L associated protein* (*clap*) and *cystatin B inhibitor* (*cstb*) was investigated through quantitative Real Time PCR (RT q-PCR), using *glyceraldehyde 3-phosphatase dehydrogenase* (*gapdh*) as housekeeping gene (Chen et al., 2009). Primers of these genes are listed in Table S2~~1~~, Supplementary Information. RT q-PCR was performed using a Stratagene Mx 3000P thermal cycler. Each amplification reaction contained PCR for each gene was performed in triplicate in a total volume of 20 μL containing 1 μL cDNA, 100 nM of each primer and 10 μL of SsoAdvanced™

Universal SYBR® Green Supermix 2x (BIORAD, Biorad Laboratories, USA). The cycling conditions were: 95°C for 2 min for polymerase activation, followed by 40 PCR cycles 10 s at 95°C; 20 s at 50-60°C, 5 s at 72°C. Cycle threshold (Ct) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. Melting curve analysis and gel electrophoresis of selected samples were performed to confirm the production of a single amplification in these reaction. Real-time efficiency of primer set (E) was determined for each gene from the slopes given by MxPro™ QPCR software (Stratagen,USA), apply the equation $E = 10^{(-1/slope)}$. The calculated relative expression ratio of each gene was based on the PCR efficiency (E) and Ct of sample compared with control, and expressed in comparison to the reference genes.

2.5. Data analysis

All statistical analysis were performed using Graphpad Prism 5. For the ecotoxicity results, normality was verified using Shapiro-Wilk test and homogeneity of variances by Bartlett's test, data were conformed to the assumptions. ~~For ecotoxicity results,~~ The one-way variance analysis (ANOVA) was performed to compare the various treatments and $p < 0.05$ was taken as significant cut-off. Results of long-term toxicity 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: $y = b + (a - b) / 1 + 10^{(Log\ LC50 - x)}$ 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.

Data obtained from gene expression analysis are expressed as mean ± standard deviation (s.d.). To compare the various treatments, ANOVA analysis was applied, using Bonferroni post-hoc and taking $p < 0.05$ (*) and $p < 0.001$ (**~~*~~) as significant cut-off.

3. Results and Discussion

The aim of the present study was to investigate the effects caused by short- and long-term exposure to nanoplastics in two planktonic species at the base of the marine trophic web, the green microalga *D. tertiolecta* and the brine shrimp *A. franciscana*, using a prey-to-predator approach. Both negatively (-COOH) and positively (-NH_2) surface charged PS NPs were used as model for nanoplastics and the different functionalization (i.e. surface charges) vs the observed toxicity was discussed.

3.1. Behaviour of PS NPs in exposure media

Nominal sizes of 40 nm for PS-COOH and 50 nm for PS-NH_2 in mQW was confirmed by TEM imaging as reported in our previous study (Bergami et al., 2016). DLS results (shown in Table 1) confirmed an optimal dispersion and stability in mQW for both PS NPs, with a Z-Average of 54.47 ± 0.82 nm for PS-COOH and 56.48 ± 0.60 nm for PS-NH_2 as well as low PDI values (0.116 and 0.161 respectively). ζ-potential values in mQW confirmed the negative surface charge (-66 ± 1.1 mV) for PS-COOH and the positive one (+53.1 ± 1.21 mV) for PS-NH_2, characteristic of PS NP functionalization.

Secondary characterization in the two exposure media (i.e. microalgae medium and NSW) confirmed the different behaviour of the two PS NPs according to their different surface functionalization. Intensity-based distributions obtained by DLS show high aggregation occurring for PS-COOH in both exposure media (Figure 1A), opposed to a better dispersion of PS-NH_2 having only few aggregates of large size (Figure 1B). Table 1 reports a comparison among PS NP parameters in the two exposure media compared to mQW, adopted as reference medium for the dispersion. Z-Average values indicate that PS-COOH originated micro-scale aggregates both in microalgae medium and NSW (as 1237.5 ± 107 nm and 1064 ± 101 nm respectively), whereas PS-

NH_2 resulted far less aggregated, with an hydrodynamic size of 127 ± 5 nm in microalgae medium and 196 ± 7 nm in NSW. Such acquired dimensions are congruent with PDI values (> 0.220) and lower absolute values of ζ-potential indicating a broader size distribution of PS NPs and instability in such exposure media, as compared to the ones in mQW.

These results confirmed our previous findings on the same batch of PS NPs dispersed in NSW (Della Torre et al., 2014; Bergami et al., 2016). NP surface charge represents one of main property driving NP behaviour in aquatic environments, in terms of stability, aggregation but also fate as mobility or deposition (Quigg et al., 2013), therefore secondary characterization is needed in ecotoxicological studies, as also recommended by SCENIHR (2007).

In general, the low stability observed in the exposure media can be due mainly to the high content of ionic salts (salinity from 30‰ in microalgae medium to 38‰ in NSW), but also to natural organic matter (NOM) and other compounds such as proteins and exopolymeric substances (EPS). naturally present in NSW. These factors may specifically trigger the transformation due to eco-interactions of the NPs in complex aquatic environments (Quik et al., 2014; Corsi et al., 2014). For instance, Kach and Ward (2008) reported that the strong aggregation of PS NPs (up to 0.5 µm) in high ionic strength media significantly reduces their bioavailability and thus limiting toxicity to aquatic organisms.

From the overall results obtained from the secondary characterization, both microalgae *D. tertiolecta* and brine shrimp *A. franciscana* in NSW were exposed to micro-aggregates (> 1000 nm) of the ~~negative~~negatively charged PS-COOH and nano-scale aggregates of positively charged PS-NH_2 (< 200 nm). These acquired new dimensions must be taken into consideration when looking at the observed toxicity.

3.2. Effects on the green microalga

The unicellular green microalga *D. tertiolecta* ~~has been~~is recommended as bioindicator for standard ecotoxicity tests (IRSA, 1978; US EPA, 1974; APHA-AWWA-WEF, 1999). In the last years, many studies regarding the effects of engineered NPs (e.g. Ag ~~NPs~~, TiO_2 ~~NPs~~ and ZnO NPs) on the growth of this model organism have been reported (Oukarrom et al., 2012; Miller et al., 2012; Hazani et al., 2013; Manzo et al., 2013). However, there is still a paucity of information on the impact of nanoplastics on marine phytoplankton, which plays a fundamental role in marine ecosystem's net primary productivity, accounting for more than an half of it. Moreover, it influences the global carbon cycle and ultimate climate by dominating the ocean and freshwater planktonic community (Field et al., 1998).

The two tested PS NPs were able to affect microalga *D. tertiolecta* growth in a different way (Figure 2A). PS-COOH was found to not significantly alter the growth of the microalgae up to 50 µg/ml (F (6, 48) = 0.7219, n.s.), with a maximum average inhibition ~~in average~~of the ~~specific~~ growth rate ($I_{\mu i}$) of 25.37% after 72 h. Similar negligible effects on the same species were reported by Sjollema et al. (2016) in term of growth inhibition and decrease in photosynthetic efficiency up to 250 µg/ml PS-COOH. Besseling et al. (2014) also reported limited effect to freshwater microalga *S. obliquus* growth by 70 nm PS-COOH, with an inhibition of 2.5% after exposure to 1 g/l PS-COOH.

The observed strong aggregation pattern of PS-COOH (Z-average > 1 µm) in the microalgae medium (Figure 1A) could be related to reduced bioavailability of nano-scale PS-COOH and ~~thus~~ explain its ~~peculiar~~ lack of toxicity, as previously reported for other aquatic species (Handy et al., 2008; Kach and Ward, 2008; Della Torre et al., 2014).

On the opposite, exposure to PS-NH_2, which were still present as NP aggregates of around 127 nm in the medium, caused a strong inhibition of the algal growth (F (6, 48) = 14.48, p < 0.0001), with an $EC_{50\ (0–72\ h)}$ of 12.97 ± 0.57 µg/ml and an $I_{\mu i}$ of 17.71~~61.02~~% at 0.5~~0~~ µg/ml. Alike, the inhibition in freshwater microalga *P. subcapitata* growth was observed by Casado et al. (2013) after exposure

to 55 and 110 nm positive surface charged PEI-modified PS NPs, with $EC_{50\ (0–72\ h)}$ values of 0.58 and 0.54 µg/ml respectively, suggesting that the amino groups on NP surface play an important role in determining the toxicity.

Considering the results obtained from the growth inhibition test, in-depth imaging analyses (i.e. fluorescent and SEM) performed on microalgae exposed to 5 µg/ml PS NPs revealed the ~~peculiar~~ specific disposition of these charged nanoplastics. Both PS NPs were clearly adsorbed on microalgae cell surface, as indicated by the yellow-green fluorescence of PS-COOH (Figure 3) and the rounded aggregates of PS-NH_2 observed by SEM (Figure 4C,D). SEM images of the control group (Figure 4A,B) show few clusters around microalgae cells withal, probably due to NOM and/or EPS present in the medium. EPS are polysaccharides that can be actively produced and released by algae concomitantly to an extracellular stress as defence mechanism (Mishra and Jha, 2009; Adeleye and Keller, 2014) to limit the penetration of foreign substances through the cell surface (Kumar et al., 2007). Since the unicellular *D. tertiolecta* lacks rigid cell wall (Oren, 2005), EPS surrounding the plasma membrane could have a major protective role against nanoplastics and particularly to PS-NH_2 (EC_{50} of 12.97 µg/ml). Therefore, it is likely that the synthesis of these biopolymers is enhanced under PS-NH_2 exposure, driving the heteroaggregation observed in Figure 4C,~~-~~D.

In addition, the observed physical adsorption of PS NPs on microalgae surface has been associated to a decrease in algal photosynthesis and ROS production in freshwater algae (Bhattacharya et al., 2010). In our study, the decrease in growth rate observed under PS NP exposure suggest that photosynthesis might be impaired and ROS production triggered in *D. tertiolecta* species. However, this phenomenon appears to be negligible for negatively charged PS-COOH (EC_{50} > 50 µg/ml), probably due to the electrostatic repulsion exerted by its carboxyl groups on microalgae cell membrane (Bhattacharya et al., 2010).

On the contrary, the adsorption of PS-NH_2 to microalgae has been reported to be dose-dependent (Bhattacharya et al., 2010) and to alter the plasma membrane by generating holes (Leroueil et al., 2008). Other studies support the "proton sponge" hypothesis, suggesting that positively ~~-~~charged NPs such as PS-NH_2 bind with high affinity to lipid bilayers on the cell membrane in favour of cellular uptake via endocytosis (Nel et al., 2009; Van Lehn and Alexander-Katz, 2011; Lin and Alexander-Katz, 2013) and thus generating toxicity (Bexiga et al., 2011; Salvati et al., 2011; Wang et al., 2013). Further studies on the green microalga *D. tertiolecta* should be addressed to stress-related responses in terms of ROS production but also EPS released upon exposure to nanoplastics.

3.3. Effects on the brine shrimp

As phytoplankton, zooplankton plays a pivotal role in marine trophic web and can be ~~considered~~ aseriously affected ~~target of~~ by nanoplastic pollution, being continuously exposed to the floating fraction of small plastic debris present in ~~the~~ surface waters (Moore, 2006; Matranga and Corsi, 2012; Frias et al., 2014).

In our previous study (Bergami et al., 2016), we emphasized how short-term exposure of *A. franciscana* larvae to PS-NH_2 (in the range 5 – 100 µg/ml) ~~was~~ could hamper~~ing~~ larvae motility and induc~~ing~~ multiple molting and the latter effect was suggested as potential defence mechanism against these positively charged nanoplastics. In this study, we decided to have an in-depth look at the molecular level behind this physiological process in order to better understand any effect over a prolonged exposure. Therefore, the expression of two genes (*clap* and *cstb*), which are known to be involved in the molting of *A. franciscana* embryos and larvae (Warner et al., 1995; Warner and Matheson, 1998; Liu and Warner, 2006), was investigated.

Figure 5 shows a significant up-regulation of *clap* ($p < 0.001$) and *cstb* ($p < 0.05$) genes in 48 h larvae exposed to 1 µg/ml PS-NH_2 with respect to the control, whereas no differences ($p > 0.05$)

were observed at the lowest concentration ~~considered~~ (0.1 µg/ml). In brine shrimp *A. franciscana*, *clap* gene codes for the non-catalytic sub-unit of the cathepsin L-like protease (Chen et al., 2009), the major cysteine peptidase involved in processes related to growth, including molting, organogenesis and tissue remodelling in early larvae (up to 44 h after hatching) (Warner et al., 1995; Warner and Matheson, 1998). Through ecdysteroid stimuli, cathepsin L-like protease is responsible for the release of serine proteases in the degradation of the cuticle detached from the epidermis (Warner and Matheson, 1998). Therefore, the strong induction of *clap* (having a normalized relative expression of 14.3) (Figure 5A) might have driven the physiological alterations previously observed in 48 h larvae (Bergami et al., 2016), suggesting a specific mechanism provoked by PS-NH_2 affecting larval molting.

The up-regulation of *cstb* gene (Figure 5B) by PS-NH_2 at 1 µg/ml could be explained as a tentative of the endogenous cystatin B inhibitor in regulating the cathepsin L-like protease activity and thus limit multiple molting, which is high energy consuming (Warner et al., 2004; Turk et al., 2012). However, the low normalized relative expression of *cstb* compared to *clap* suggests that a long-term exposure to PS-NH_2 could seriously affect brine shrimp growth, physiology and survival, as previously hypothesized (Bergami et al., 2016).

In order to verify this hypothesis, brine shrimp larvae of *A. franciscana* were exposed to ~~negative~~negatively -PS-COOH and positively charged PS-NH_2 for 14 d and fed with microalgae *D. tertiolecta*.

The results of long-term toxicity test are reported in Figure 2B as % of mortality to increasing concentrations of PS NPs. The average mortality observed in the control group was 10 ± 3.25% and 14 ± 6.81% for PS-COOH and PS-NH_2 experiments respectively (mean ± s.d.). Even in 14 d exposure scenarios, micro-scale aggregates of PS-COOH did not significantly affect brine shrimps up to 10 µg/ml ($F(2, 17) = 1.112$, n.s.) confirming previous findings on short-term negligible effects. On the contrary, PS-NH_2 resulted better dispersed in the medium and caused a dose-

dependent toxicity (LC_{50} of 0.83 µg/ml, IC 95%: 0.07 – 0.94) after 14 d. ~~,~~A significant difference respect to the control group was found for treatments above 1 µg/ml PS-NH_2 ($F(6, 62) = 84.51$, $p < 0.0001$) and all ~~with~~ the exposed organisms were ~~100% of~~ found dead ~~organisms~~ at concentrations above 5 µg/ml. The strong effect observed could be related to PS-NH_2 low aggregation in NSW, having an hydrodynamic size of 196 nm (Figure 1B) and therefore being dispersed and bioavailable to planktonic species (Kach and Ward, 2008; Della Torre et al., 2014).

These toxicity data are more informative with respect to those from the short-term toxicity test (48 h), where no significant mortality up to 100 µg/ml was found (Bergami et al., 2016). Therefore, for this species the long-term exposure was crucial to discriminate the different toxicity between negatively and positively surface charged PS nanoplastics.

The higher sensitivity of brine shrimp larvae to toxicants in long-term exposure conditions compared to acute and hatching tests has been recently reported by Manfra et al. (2015) and Rotini et al. (2015), as a consequence of integration of responses at different brine shrimp larval stages over an extended period of time. Moreover, from an ecotoxicological point of view, in order to assess the hazard posed by nanoplastics, the long-term test mimics more likely environmental realistic scenarios (Lenz et al., 2016) and at the same time represents two trophic levels.

In terms of particles disposition, optical fluorescent microscopy showed yellow-green fluorescent PS-COOH aggregates of an average size of 1 µm (Table 1) both in brine shrimp digestive tract (Figure 6A,B) as well as faecal pellets (Figure 6C,D). Such disposition resembles the one observed in acute exposure (Bergami et al., 2016), ~~and is~~ in agreement with studies performed on other zooplanktonic species (Cole et al., 2013; Lee et al., 2013; Della Torre et al., 2014). Although it has been hypothesized that plastic particles in the digestive tract could hamper the feeding, inhibit the digestion and reduce physiological functions after prolonged period (Besseling et al., 2014; Cole et al., 2013; Lee et al., 2013). Nevertheless, in this study the accumulation of PS-COOH seemed not affecting the survival of brine shrimps up to 10 µg/ml. Nonetheless, processes of trophic web

transfer (biomagnification) cannot be excluded upon the evidence of nanoplastic ingestion by marine zooplankton, as already hypothesized by Cerdevall et al. (2012) and Mattson et al. (2015). In addition, considering the ability of PS NPs to adsorb hydrophobic contaminants (Rios et al., 2007; Rochman et al., 2013, Velzeboer et al., 2014), their uptake and sequestration in the gut of exposed organisms may indicate dramatic consequences for ~~the~~ marine organisms.

Furthermore, the dose-dependent mortality observed validates ~~our~~ the hypothesis we formulated in our previous study (Bergami et al., 2016) regarding the long-term toxicity of PS-NH_2~~(Bergami et al., 2016),~~ – in line with the results from gene expression showing a significant *clap* and *cstb* modulation in 48 h larvae at 1 µg/ml PS-NH_2 (Figure 5). The long-term toxicity test is a semi-static assay where solutions are renewed every 2-3 days (Savorelli et al., 2007; Manfra et al., 2012), thus the strong induction of *clap* gene ~~after~~ in 48 h ~~in early~~ larvae indicates that PS-NH_2~~, over a prolonged exposure,~~ is able to disrupt the physiology and the energy flow in brine shrimp developing larvae over a prolonged exposure.

Optical microscopy images of 9 d brine shrimps exposed to high concentrations (5 and 10 µg/ml) of PS-NH_2 (Figure 7B) support these findings, showing that the exposed organisms were not able to reach the same larval stage of the control group (Figure 7A). However, no measurement of larvae length was made at this stage (9 d) and~~, since~~ all the ~~exposed~~ organisms exposed at these concentrations were found dead ~~at these concentrations~~ after 14 d. Likewise, at low~~er~~ concentrations (~~<~~ 0~~2~~.5 and 1 µg/ml), no significant difference in total length of PS-NH_2 exposed organisms versus control was observed ($p > 0.05$) (Figure S1). Accordingly, when *Artemia* long-term toxicity test was proposed (Manfra et al., 2012), the authors underlined that growth (~~carapace~~ i.e. length after 14 d) as sub-lethal endpoint was far less sensitive compared to mortality.

In the last decade, cysteine cathepsins have been ~~also~~ associated to apoptotic signalling as a consequence of lysosomal destabilization and leakage caused by ROS (Chwieralski and Bühling, 2006). The release of cathepsines into the cytoplasm can be related to a cascade of intracellular

degradative events that can lead to a direct process of apoptosis or promoting mitochondria ROS generation and amplifying the activation of caspases (Chwieralski et al., 2006; Turk and Stoka, 2007; Turk et al., 2012).

In our study, the strong induction of *clap* gene encoding for cathepsin L-like protease in response to PS-NH_2 ~~in brine shrimp larvae~~ confirms the apoptotic pathway already observed in our previous studies in sea urchin embryos and mussel's hemocytes (Della Torre et al., 2014; Canesi et al., 2015; Pinsino et al., 2017) and in studies with human cell lines (Wang et al., 2013) for the same particle~~upon PS-NH_2 exposure~~.

Regarding the mechanism of toxicity behind PS-NH_2, different hypotheses can be formulated: (I) a direct toxicity caused by the prolonged exposure up to 14 d with strong up-regulation of *clap* and *cstb* genes and consequently disruption of larval molting and energetic metabolism; (II) indirect toxicity, driven by the strong physical adsorption of positively charged nanoplastics on microalgae, leading to uptake and toxicity to ~~the~~ brine shrimps once they feed on microalgae; (III) a combination of direct and indirect toxicity as integration of prey-to-predator responses.

Overall, by comparing LC_{50} values from 14 d ~~exposure of~~ brine shrimps with E(L)C_{50} from other marine model organisms exposed to the same PS NPs (Table 2), it can be noted a similar pattern of toxicity among different species, likely related to the surface charge of the nanoplastics, with absence of toxicity for PS-COOH opposed to strong toxic effects provoked by PS-NH_2. The long-term toxicity test can be seen as the integration of ~~prey-to-predator~~different responses and~~,~~ appears to be a sensitive means to determine nanoplastics toxicity. ~~Therefore, t~~The use of long-term end-point should be included in the best practices for determining the impact of emerging contaminants in the marine environment, such as nanoplastics and engineered nanomaterials.

4. Conclusion

Nanoplastic exposure and toxicity constitute one of the main unexplored areas of plastic pollution ~~last frontier~~ to fully understand the magnitude and consequences of this threat ~~plastic pollution and its consequences for~~ to the marine environment. This study investigates the effects of ~~model~~ negatively (-COOH) and positively (-NH_2) surface charged PS NPs to marine planktonic species at low tested concentrations and in a prolonged exposure scenario. Green microalga *D. tertiolecta* and brine shrimp *A. franciscana* were chosen as model organisms representing two levels at the base of the marine trophic web, as prey and predator respectively. PS-COOH formed micro-scale aggregates in the media and did not affect the growth of microalgae up to 50 µg/ml as well as brine shrimps up to 10 µg/ml. However, these ~~negative~~negatively charged NPs were found adsorbed on microalgae as well as accumulated and excreted in brine shrimps, suggesting a potential trophic transfer along marine trophic webs. On the opposite, PS-NH_2 was found as nanometric aggregates in both media, causing inhibition of algal growth (EC_{50} = 12.97 µg/ml) and mortality in brine shrimps at 14 d (LC_{50} = 0.83 µg/ml). Moreover, 1 µg/ml PS-NH_2 significantly induced *clap* and *cstb* genes, explaining the physiological alterations previously observed in 48 h larvae, but also suggesting an apoptotic pathway triggered by cathepsin L-like protease in brine shrimps continuously exposed to positively charged nanoplastics. Eventually, the mortality at 14 d was critical to discriminate PS NP toxicity to brine shrimp, being also representative of the interactions occurring between the nanoplastics and the organisms during the exposure ~~two trophic levels~~. Overall, our findings indicate that PS NP surface chemistry is a key parameter responsible for the behaviour, eco-interactions and impact on marine phyto- and zooplankton in terms of adsorption, accumulation and toxicity. The use of long-term end-point has been identified as valuable tool for determining the impact of nanoplastics on brine shrimp.

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Tables with caption

Table 1. Physico-chemical parameters of PS NPs in milli-Q water (mQW, T = 20°C), microalgae medium (prepared in NSW 0.45 µm filtered, T 20°C, salinity 30‰, pH 8.3) and natural sea water (NSW, 0.45 µm filtered, T 25°C, salinity 38‰, pH 8.3) using DLS analysis. Z-average (nm), polydispersity index (PDI) and ζ-potential (mV), referred to PS NP concentration of 50 µg/ml are reported. Values are shown as average ± standard deviation of 3 measurements.

	40 nm PS-COOH			50 nm PS-NH_2		
	Z-Average (nm)	**PDI**	**ζ-potential (mV)**	**Z-Average (nm)**	**PDI**	**ζ-potential (mV)**
mQW	54.47 ± 0.82	0.116 ± 0.03	- 66 ± 1.1	56.48 ± 0.60	0.161 ± 0.014	+ 53.1 ± 2.1
microalgae medium	1237.5 ± 107	0.226 ± 0.03	- 12.4 ± 1.7	127 ± 5.07	0.316 ± 0.033	+ 16.5 ± 3.1
NSW	1064.2 ± 100.7	0.241 ± 0.07	- 9.18 ± 2.3	196 ± 7.46	0.287 ± 0.01	+ 17.5 ± 1.4

Table 2. EC_{50} or LC_{50} values (µg/ml) for different marine organisms belonging to phyto- (green microalga *D. tertiolecta*) and zooplankton (sea urchin *P. lividus* embryos and brine shrimp *A. franciscana* larvae), exposed to 40 nm negatively PS-COOH and 50 nm positively PS-NH_2 charged nanoplastics in NSW media.

Species	**Test**	**PS-COOH**	**PS-NH_2**	**Reference**
P. lividus	Embryotoxicity (48 h)	EC_{50} > 50 µg/ml	EC_{50} = 2.61 µg/ml	Della Torre et al. (2014)
D. tertiolecta	Growth Inhibition (72 h)	EC_{50} > 50 µg/ml	EC_{50} = 12.97 µg/ml	present study
A. franciscana	Acute Toxicity (48 h)	LC_{50} > 100 µg/ml	LC_{50} > 100 µg/ml	Bergami et al. (2016)
	Long-term Toxicity (14 d)	LC_{50} > 10 µg/ml	LC_{50} = 0.83 µg/ml	present study

Figures with caption

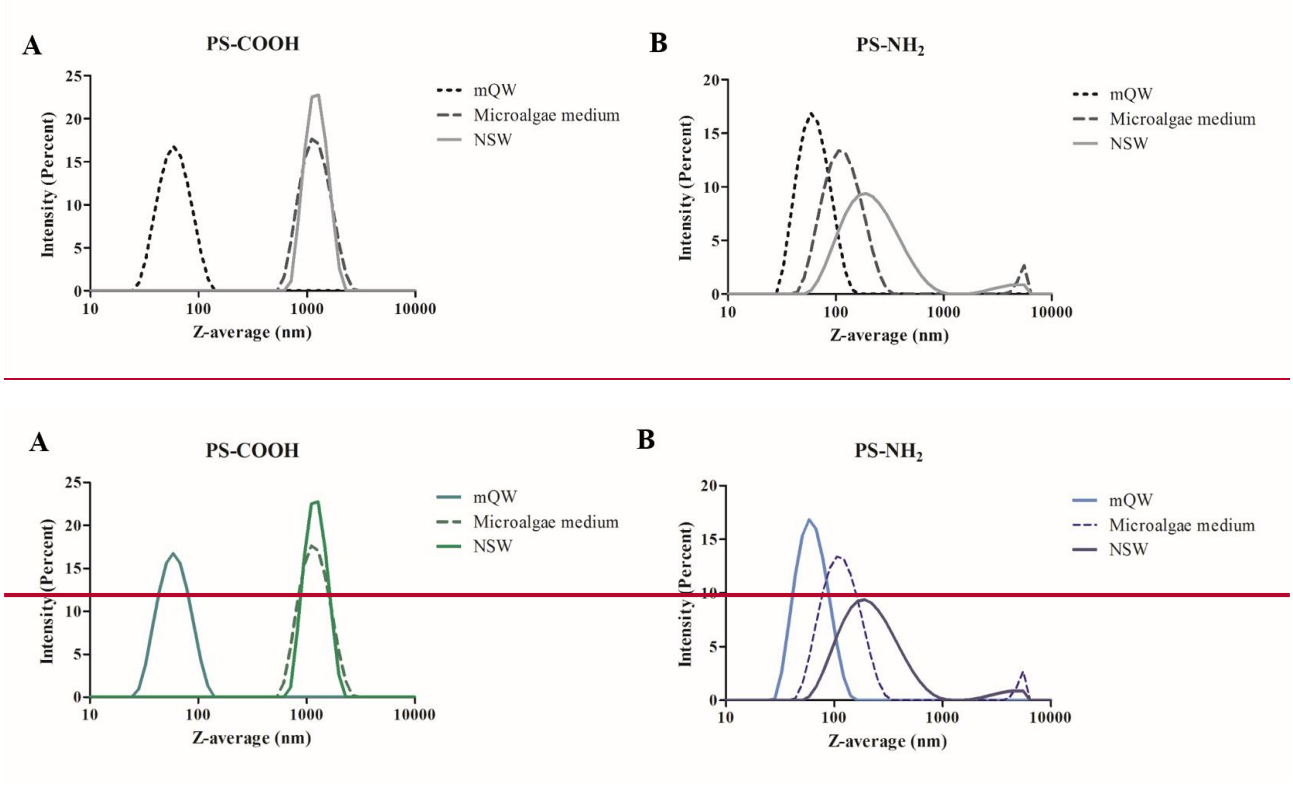
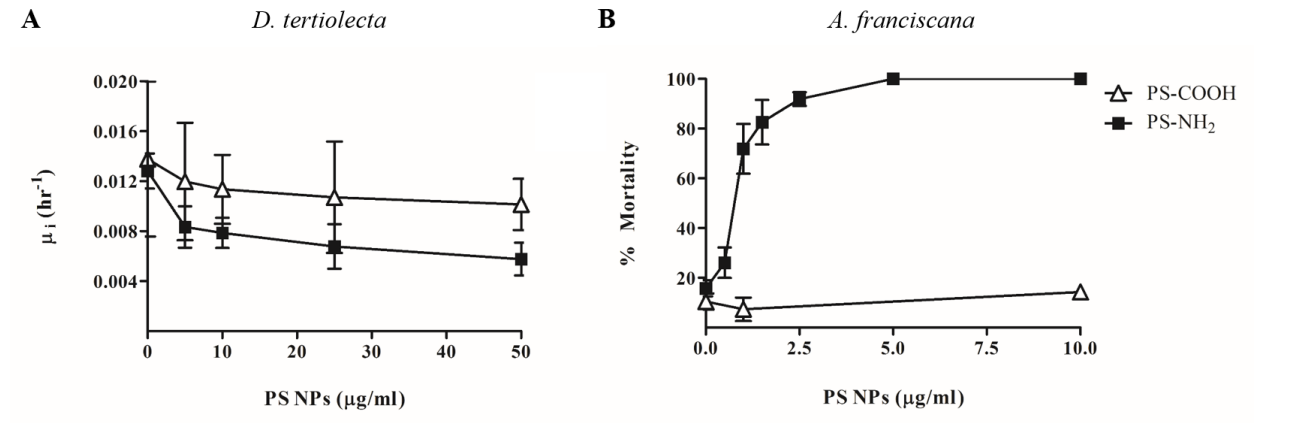


Figure 1. Intensity-based size distributions by DLS analysis of 40 nm PS-COOH (A) and 50 nm PS-NH_2 (B) at 50 µg/ml in milli-Q water (mQW), natural sea water (NSW) and microalgae medium. For each medium, one ~~indipendent~~independent representative measurement is reported. The X-axis showing Z-average (nm) is set at 10 nm, logarithmic scale. Graphs were edited using GraphPad Prism5.



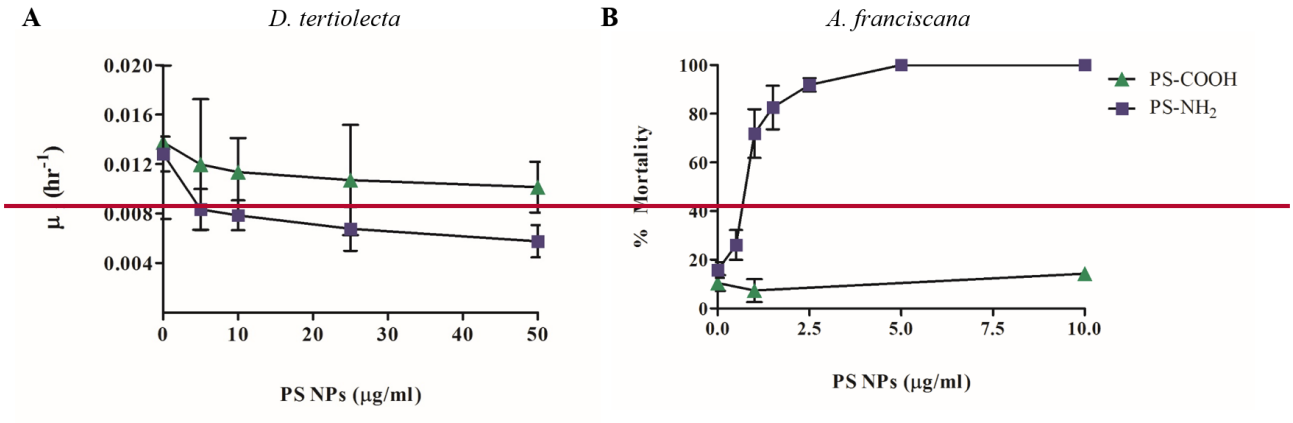


Figure 2. Ecotoxicity of PS NPs to plaktonic organisms. Average growth rate (μ_i, hr^{-1}) of green microalga *D. tertiolecta* after 72 h of exposure to increasing concentrations of PS NPs ~~(growth inhibition test)~~ (A, growth inhibition test). Values corresponding to 0 – 5 – 10 – 25 – 50 µg/ml are reported. Mortality (%) of brine shrimp *A. franciscana* after 14 d of exposure to increasing concentrations of PS NPs. Control groups showed an average mortality of 10±3.25% and 14±6.81% for PS-COOH and PS-NH_2 experiments respectively ~~(long-term lethal toxicity test)~~ (B, long-term lethal toxicity test). Error bars indicate standard deviation. Graphs were edited using GraphPad Prism5.

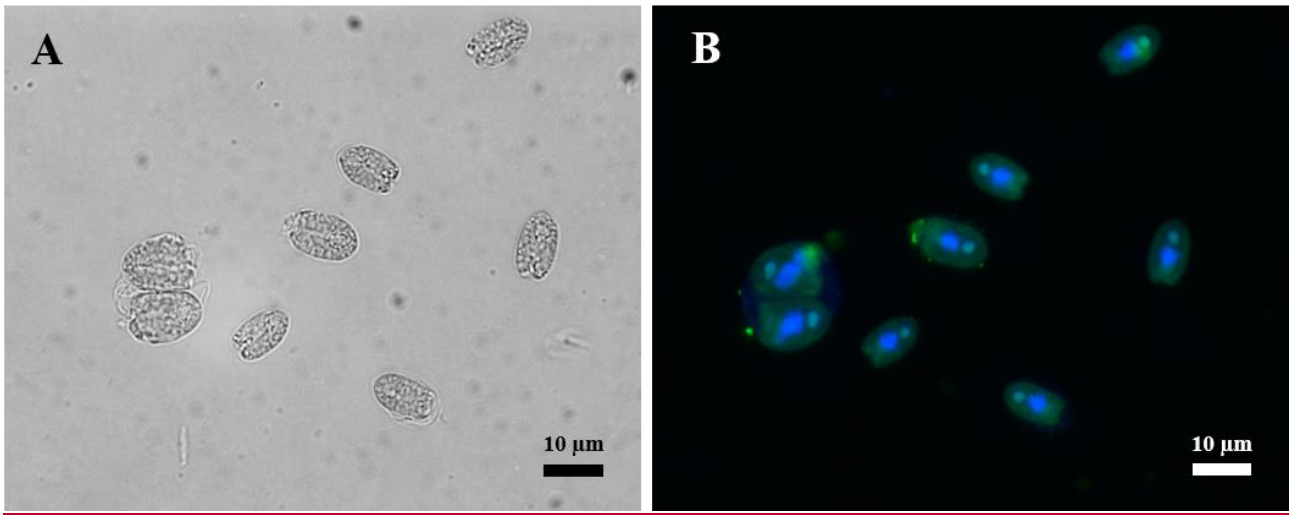


Figure 3. Disposition of yellow-green fluorescent PS-COOH at 5 µg/ml in green microalga *D. tertiolecta* (growth inhibition test) by optical (A) and fluorescent (DAPI+FITC filter) (B) microscopy. Microalgael ~~el~~ nuclei were stained with DAPI (blue). Images were taken at 63X. Scale bar: 10 µm.

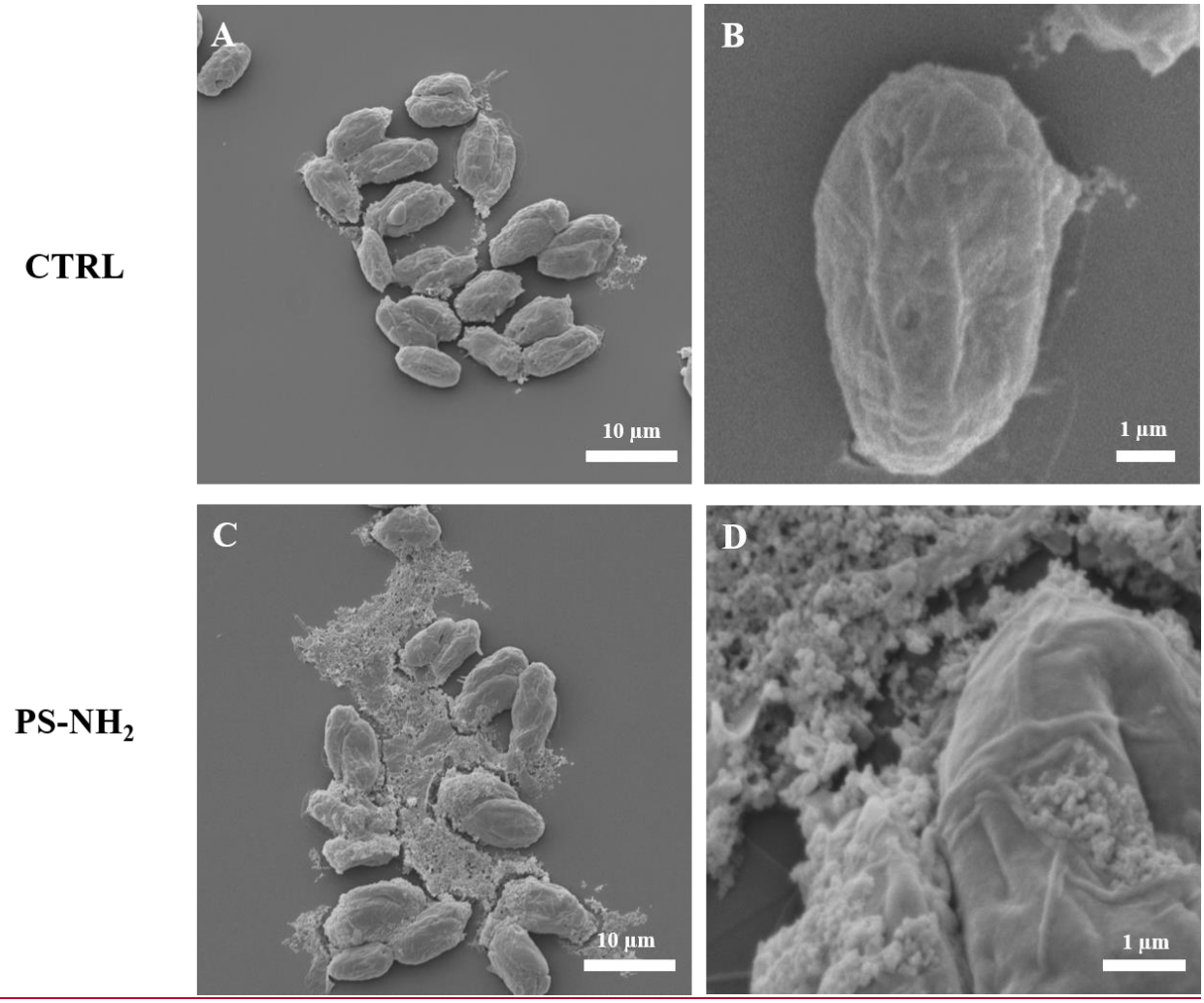
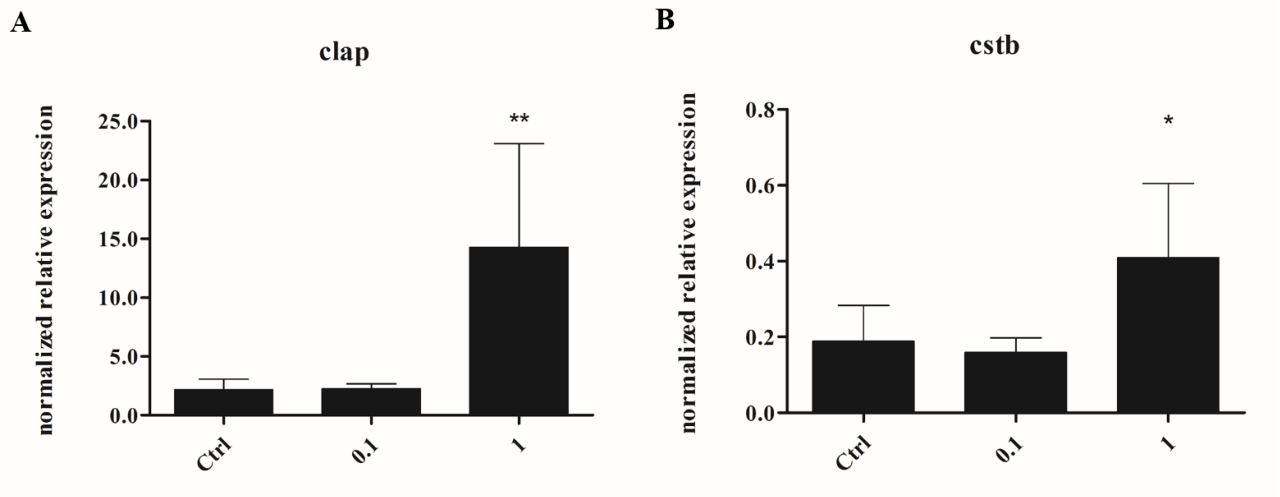


Figure 4. Disposition of unlabelled PS-NH_2 in green microalga *D. tertiolecta* (growth inhibition test). SEM images showing control (A, B) and exposed to 5 µg/ml- PS-NH_2 (C, D). Images were taken at 1600X (A, C), 6400X (B) and 12800X (D). Scale bar: 10 µm (A, C) and 1 µm (B, D).



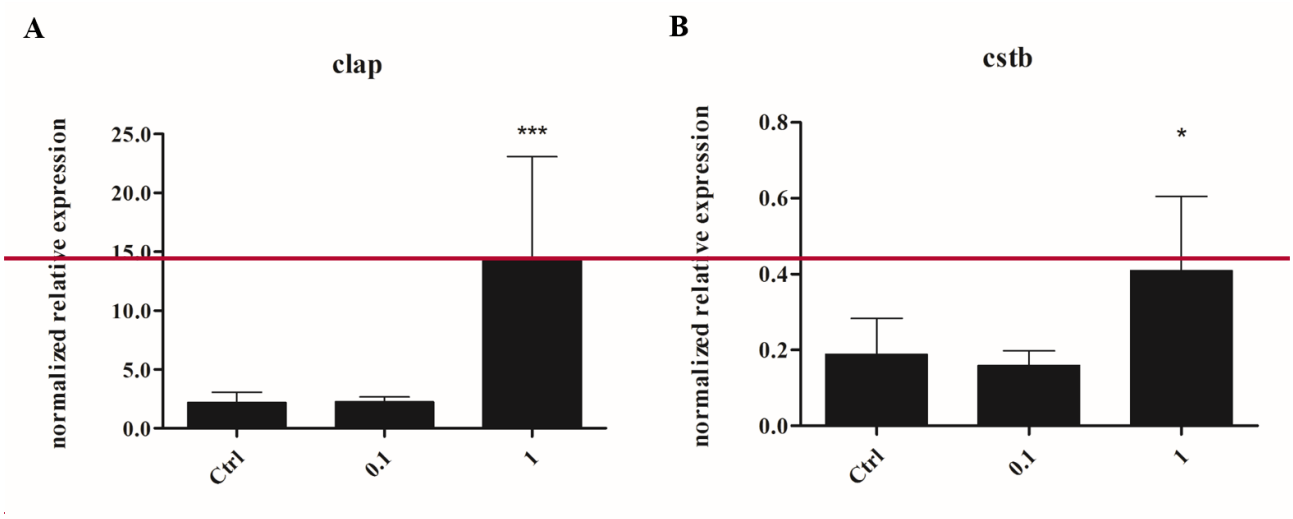


Figure 5. Expression of *clap* (A) and *cstb* (B) genes in brine shrimp *A. franciscana* larvae after short-term exposure (48 h) to positive surface charged PS-NH_2 at 0, 0.1 and 1 µg/ml. Results are shown as mean ± standard deviation. *~~*~~* and * indicate~~s~~ significant differences respect to the control group, corresponding to $p < 0.001$ and $p < 0.05$ respectively.

Figure 6. Disposition of yellow-green fluorescent PS-COOH at 10 µg/ml in brine shrimp *A. franciscana* larvae after 14 days of exposure (long-term toxicity test). Fluorescent aggregates were observed inside the digestive tract (A, B), but also excreted as fecal pellets (C, D). Scale bar: 500 µm.

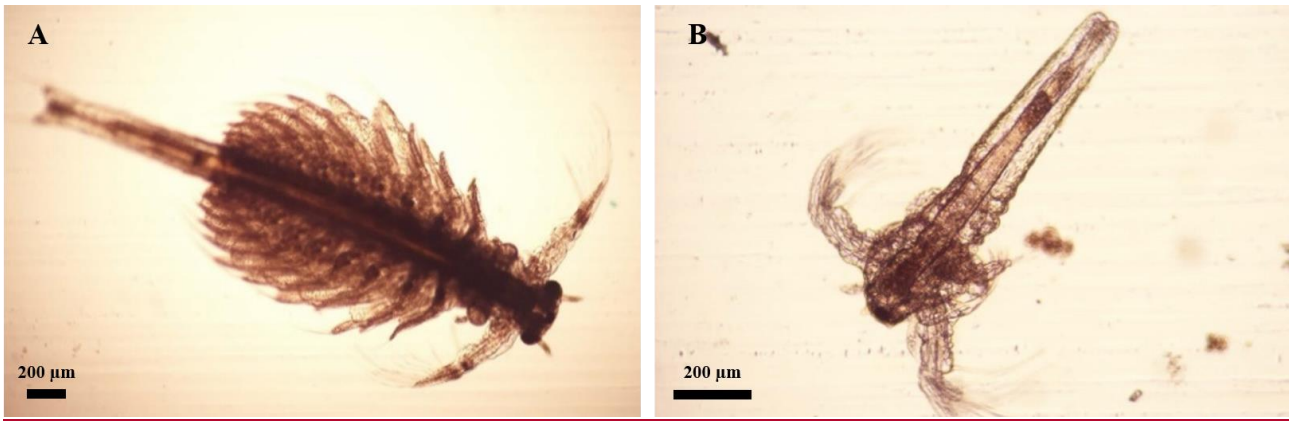


Figure 7. Effect of PS-NH_2 at 10 µg/ml on brine shrimp *A. franciscana* growth (B), compared to the control group (A) after 9 days of exposure (long-term toxicity test). Scale bar: 200 µm.

Long-term toxicity of surface charged polystyrene nanoplastics to marine planktonic species *Dunaliella tertiolecta* and *Artemia franciscana*

Bergami E.[1*], Pugnalini S.[1], Vannuccini M. L.[1], Manfra L.[2, 3], Faleri C.[4], Savorelli F.[5], Dawson K. A.[6], Corsi I.[1]

[1]Department of Physical, Earth and Environmental Sciences, University of Siena, Italy;

[2]Institute for Environmental Protection and Research (ISPRA), Rome, Italy;

[3]Department of Biology and Evolution of Marine Organisms, Anton Dohrn Zoological Station, 80121 Naples, Italy;

[4]Department of Life Sciences, University of Siena, 53100 Siena, Italy;

[5]Regional Agency for Prevention, Environment and Energy of Emilia-Romagna (Arpae ER), Ferrara, Italy

[6]Centre for BioNano Interactions, School of Chemistry and Chemical Biology, University College Dublin, Ireland;

***Corresponding author:** Elisa Bergami; bergami@student.unisi.it; Department of Physical, Earth and Environmental Sciences, University of Siena, Italy.

Supporting Information

The following two pages of supporting information contains two tables associated to this manuscript.

Materials and Methods

Polystyrene nanoparticles

Table S1. Conversion chart showing concentrations (µg/ml) and numbers (NPs/ml) of the nanoplastics adopted in this study. The particle numbers were calculated using the equations obtained from the suppliers (see FluoSpheres® Fluorescent Microspheres and TechNote 206) and considering the nominal size of the NPs (40 nm PS-COOH from Invitrogen and 50 nm PS-NH_2 from Bangs Laboratories Inc.).

PS NPs µg/ml	PS-NH_2 NPs/ml	PS-COOH NPs/ml
50 µg/ml	$7.31 \cdot 10^{11}$	$1.42 \cdot 10^{12}$
25 µg/ml	$3.64 \cdot 10^{11}$	$7.11 \cdot 10^{11}$
10 µg/ml	$1.46 \cdot 10^{11}$	$2.84 \cdot 10^{11}$
5 µg/ml	$7.28 \cdot 10^{10}$	$1.42 \cdot 10^{11}$
2.5 µg/ml	$3.64 \cdot 10^{10}$	$7.11 \cdot 10^{10}$
1.5 µg/ml	$2.18 \cdot 10^{10}$	$4.27 \cdot 10^{10}$
1 µg/ml	$1.46 \cdot 10^{10}$	$2.84 \cdot 10^{10}$
0.5 µg/ml	$7.281 \cdot 10^{9}$	$1.42 \cdot 10^{10}$

RNA extraction, cDNA synthesis and Real Time q-PCR

Table S2~~1~~. Prime sequences, length, accession number and annealing temperature for brine shrimp *A. franciscana* genes investigated through RT-qPCR analysis in this study. While *cstb* and *gapdh* sequences were already published (Chen et al., 2009), primers for *clap* were designed using NCBI Primer-BLAST.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Acc number	Annealing T (°C)
clap	AGCACGACATGGAACAGTGA	GCATCGTGGTTCCCTCCATT	AY307377.2	57
cstb	GCGAGAAGTCTTATCAAGT	TCTTTTACAGGAGTGATGG	Chen et al. (2009)	52
gapdh*	GTTGATGGCAAACTCGTCATA	CCACCTTCCAAGTGAGCATTA	Chen et al. (2009)	55

*housekeeping gene

Results

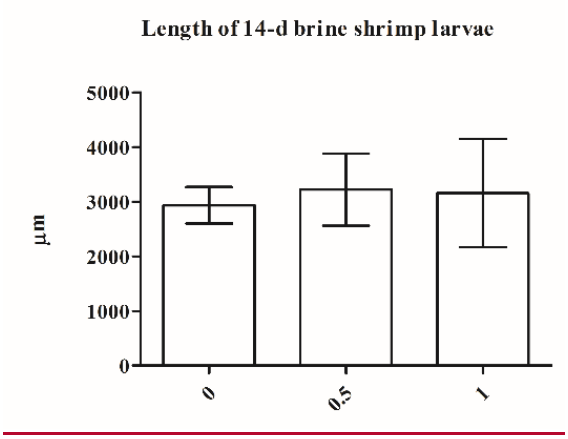


Figure S1. Brine shrimp length after 14 days of exposure to PS-NH_2 (at 0.5 and 1 µg/ml) compared to the control group. Results are expressed as means ± s.d. and representative of three independent experiments. No significant difference among the groups (One-way ANOVA, $p > 0.05$) was observed. The length of the organisms was measured from the cephalic region (i.e. nauplius eye) to the telson.

References

Chen, W-H., Ge, X., Wang, W., Yu, J., Hu, S., 2009. A gene catalogue for post-diapause development of an anhydrobiotic arthropod *Artemia franciscana*. BMC Genomics 10: 52. DOI: 10.1186/1471-2164-10-52.

FluoSpheres® Fluorescent Microspheres (Molecular Probes): https://www.google.it/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&cad=rja&uact=8&ved=0ahUKEwiwwf3T8oXUAhXJXhoKHVykCrIQFgg7MAE&url=https%3A%2F%2Ftools.thermofisher.com%2Fcontent%2Fsfs%2Fmanuals%2Fmp05000.pdf&usg=AFQjCNE_N1Hep7jhmxJchJJbbQ_hzk8-tg

NCBI Primer-BLAST http://www.ncbi.nlm.nih.gov/tools/primer-blast/

TechNote 206 (Bangs Laboratories Inc.): www.bangslabs.com/sites/default/files/imce/docs/TechNote%20206%20Web.pdf