Time-dependent effects of polystyrene nanoparticles in brine shrimp *Artemia franciscana* at physiological, biochemical and molecular levels

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Key words: Polystyrene nanoparticles, *Artemia franciscana*, Toxicity, Biomarkers

Highlights

1. Growth and development in 48 h nauplii exposed to PS-NH_2 was affected in a dose-dependent manner
2. Survival, but not growth and feeding behavior was impaired in juveniles exposed to PS-NH_2
3. Neurotoxicity and oxidative stress were observed after exposure to PS-NH_2
4. Genes involved in cell protection, development and molting were modulated at 1 μg/mL PS-NH_2

Abstract

Micro- (< 5 mm) and nanoplastics (< 1 μm) are emerging threats for marine ecosystems worldwide. Brine shrimp *Artemia* is recognized as a suitable model among planktonic species for studying the impact of polystyrene nanoparticles (PS NPs) through short and long-term bioassays. Our study aims to evaluate the time-dependent effects of cationic amino-modified PS-NH_2 (50 nm) in *A. franciscana* after short- (48 h) and long-term exposure (14 days). For this purpose, nauplii were exposed to a concentration range of PS-NH_2 (0.1, 1, 3 and 10 μg/mL) in natural sea water (NSW), and physiological, biochemical and molecular responses were investigated. Short-term exposure to PS-NH_2 caused a decrease in nauplii growth and affected the development in a concentration-dependent manner, long-term exposure impaired the survival, but not the growth and feeding behavior. Oxidative stress was detected after short term exposure as the decrease in the activity of antioxidant enzymes, and was fully evident in the long-term as lipid peroxidation, suggesting an accumulative effect. The decrease in Cholinesterase (ChE) activity observed indicates possible neurotoxic action of PS-NH_2. Also, Carboxylesterase (CbE) inhibition by PS-NH_2, described for the first time in this study, anticipates potential effects in biotransformation of exogenous and endogenous compounds, being the crustacean juvenile hormone methyl farnesoate (MF) that regulates development and molting, one candidate. Furthermore, short- and long-term exposure to PS-NH_2 affect the expression of genes involved in cell protection, development and molting. Overall, our results reveal that low PS-NH_2 concentrations induce physiological, biochemical and molecular (changes in gene expression) alterations in *Artemia*, and point at their potential risk for this model organism, supporting the general concern about nanoplastics occurrences in aquatic environments and their ability to represent an ecological threat for aquatic zooplanktonic species.

1. Introduction

The presence of marine litter on beaches and in world' oceans is a significant and pervasive global problem (Galgani et al., 2015). Plastics represent up to 95% of marine litter on shorelines, sea surface and seafloor (Galgani et al., 2015) and enter the marine environment largely as unintended wastes due to past and current disposal practices (Jambeck et al., 2015). It has been estimated that between 4.8 to 12.7 million metric tons of plastic waste enters the oceans annually. This amount is far higher than concentrations of floating plastic debris reported globally and it is expected to increase by an order of magnitude by 2025 (Jambeck et al., 2015). Plastic pathways from land to the sea have been mainly identified. Once in the sea, plastics undergo processes which will strongly affect their ability to be buoyant or sink to the seafloor (Cole et al., 2016). The smallest fractions of plastic debris identified as microplastics and nanoplastics, below 5 mm and 1µm in size respectively (Hartmann et al., 2019), can originate from weathering and fragmentation of larger debris, although other origins from nano-enabled commercial products cannot be excluded (Koelmans et al., 2015). Macro-, meso- and microplastics have largely been documented to accumulate in five oceanic gyres (Cózar et al., 2014; Eriksen et al., 2014). However, the occurrence of nanoplastics have been documented only recently in the Western North Atlantic Ocean subtropical gyre, providing the first evidence of their occurrence in surface waters (Ter Halle et al., 2017).

According to the model of Kooi et al. (2016), smallest plastics end up to be buoyant and mostly occurring in the first 0-3 meters of the water column, therefore posing a more serious threat to plankton and its predators. Nanoplastics share the peculiar properties of nanomaterials (e.g. nm size, high surface/volume ratio, high reactivity) which are able to interact with cells and living organisms in a different fashion compared to micro-sized materials and molecules. Such properties are related to uptake and toxicity of nanoscale particles (Salvati et al., 2011; Fröhlich et al., 2012; Bexiga et al., 2014; Lehner et al., 2019). Recent ecotoxicity studies have shown that polystyrene nanoparticles/nano-spherules (PS NPs) cause mild to severe impacts on a range of organisms often as a function of surface functionalization (Della Torre et al. 2014; Canesi et al. 2015, 2016; Bergami et al. 2016, 2017; Manfra et al. 2017; Pinsino et al. 2017; Marques-Santos et al., 2018; Mishra et al., 2019). Several studies have also reported the ingestion of nanoplastics in euryhaline and marine zooplankton (Della Torre et al. 2014; Manfra et al., 2017; Bhargava et al., 2018; Tallec et al.,

2018) under laboratory-controlled conditions.

PS is one of the polymers commonly found in marine litter, together with polyethylene, polypropylene and polyvinylchloride (Suaria et al., 2016). Therefore, micro- and nano-sized plastics originated from these polymers are likely to occur in surface waters and their abundance may reflect the predicted environmental concentrations (in the range of µg/L) or be much higher in particularly polluted areas (Al-Sid-Cheik et al., 2018). Nanoplastics put plankton under threat and more efforts should be posed to design relevant exposure assays to evaluate the impact on primary producers and consumers, which represent key ecological components for ocean productivity and overall carbon pump.

Brine shrimp *Artemia* has been largely used for acute and chronic toxicity testing as aquatic model organism in ecotoxicological studies (Persoone and Wells, 1987; Nunes et al., 2006; Varó et al., 2015), including nanomaterials (Ates et al., 2013; Bergami et al 2016; 2017; Gambardella et al., 2014; Manfra et al. 2017; Mishra et al., 2019). Furthermore, a new standardized testing procedure for nanomaterial environmental toxicity in hypersaline ecosystems (International Organization for Standardization/Technical Specification (ISO/TS) 20787) has been recently published using *Artemia* sp. as aquatic model organism (Johari et al., 2018).

Our previous findings on nauplii of *A. franciscana* (Bergami et al., 2016), clearly show an impairment in swimming and a significant increase in molting events upon short -term exposure (48 h) to 50 and 100 µg/mL of amino-modified PS NPs (PS-NH_2). However, no effect on mortality was observed using the standard acute toxicity test LC50 < 100 µg/mL (Artoxkit, 2014). Also, an increase in the expression of genes related with growth (*clap*) and molting (*cstb*) was observed in nauplii after 48 h exposure to 1 µg/mL of PS-NH_2 (Bergami et al. 2017). In the same study, a long-term toxicity test after 14 days upon exposure to PS-NH_2 showed a LC_{50}= 0.83 µg/mL highlighting the importance of the long-term studies when assessing the impact of nanoplastics on zooplanktonic species.

Our hypothesis is that the differences between short- and long-term toxicity to PS-NH_2 exhibited by *Artemia* are related to changes in the time-dependent responses to these nanoparticles at physiological, biochemical and molecular level.

To test this hypothesis, we evaluated the specific physiological, biochemical and molecular alterations caused by PS-NH_2 under short- (48 h) and long-term exposure conditions (14 days). Growth, development and feeding behavior were determined as

physiological endpoints. Processes, such as biotransformation, neuronal transmission, oxidative stress and stress response, were investigated through carboxylesterases (CbE) and cholinesterases (ChE) activities, glutathione-S-transferase (GST) and catalase (CAT) activities, lipid peroxidation (LP) and heat shock protein (HSP). At the molecular level, the expression of selected genes involved in stress response (*hsp26*, *hsp70*), metabolism and biosynthesis (*cystatin B inhibitor* (*cstb*), *cathepsin L-like protease* (*clap*), *chaperonin-containing TCP* (*tcp*)) during brine shrimp development was also determined.

2. Materials and methods

2.1 Behavior of PS-NH_2 in natural sea water (NSW)

Unlabeled cationic amino-modified polystyrene PS nano-spheres (PS-NH_2, nominal size of 50 nm) were purchased from Bangs Laboratories Inc. The stock solution contained 100 mg/mL of PS-NH2 in milliQ water (mQW) and was kept at 4°C until use. The same batch corresponding to Bergami et al. (2017) and Manfra et al. (2017) was used. Primary size and shape of PS-NH_2 in mQW as well as aggregation in the exposure medium after 48 h of incubation were determined through Transmission Electron Microscopy (TEM) analysis, as reported in Manfra et al. (2017). For characterization purposes, NP stock was sonicated by ultrasonic bath (CEIA CP316 digit, maximum power 600W, nominal frequency 40kHz, amplitude 90%) for 5 min at ambient temperature and vortexed. PS-NH_2 working solutions (50 μg/mL) were then prepared diluting the stock solution in mQW or natural sea water (NSW 0.20 μm filtered, T = 25°C, salinity 38.3 g/L, pH 8.25, conductivity of 57.5 ms/cm), the latter collected along Torre la Sal coastal area (East of Spain, 40º07'39''N 000º09'59''E) and vortexed prior the analysis.

Physico-chemical characterization of PS-NH_2 was performed using Dynamic Light Scattering (DLS, Malvern instruments), combined with the Zetasizer Nano Series software, version 7.02 (Particular Sciences, UK). The instrument allows the determination of key NP parameters, such as Z-average (nm), polydispersity index (PDI, dimensionless) and Zeta (ζ-)potential (mV), referred to average hydrodynamic diameter, size distribution and surface charge respectively.

Three independent measurements, each containing 11 runs of 10 second for Z-average and PDI and 20 runs for ζ-potential were performed. The stability of PS-NH_2 during the experimental assays, thus after 48 h and 72 h corresponding to the renewal of the

solutions in the long-term assay (see section 2.3), was further determined by DLS. PS-NH_2 solutions (50 μg/mL) in mQW and NSW were kept under controlled static conditions (25± 0.5 ºC, photoperiod 16:8 h light:dark) in a thermostatic chamber and analyzed by DLS.

2.2 Model organism

Newly hatched nauplii of *A. franciscana* were obtained from commercially dried cysts (INVE Company, Belgium). The hatching was carried out following Comeche et al. (2017) procedure, with some modifications reported below. Briefly 0.1 g of dry cysts were placed in a cylinder glass tube with conical bottom filled with 1 L of NSW (38 g/L), inside a thermostatic bath at 28°C with constant aeration and illumination (1000 lux). After 22 h, newly hatched nauplii (Instar I) were collected by siphoning in a 150 μm mesh, washed with NSW and transferred to a Petri dish filled with 0.2 μm filtered NSW. Then, nauplii were separated from any remaining shells and unhatched cysts based on their positive phototaxis.

2.3 Short- and long-term experimental assays

Working solutions of PS-NH_2 used for ecotoxicological tests were prepared in 0.2 μm filtered NSW from a stock of 10 mg/mL in mQW, which was sonicated by ultrasonic bath (see 2.1 for details) before dilution.

To evaluate the effect of PS-NH_2 on brine shrimp nauplii after short-term exposure (48h), batches of ≈200 newly hatched nauplii (Instar I) were exposed to increasing concentrations of PS-NH_2 (0.1, 1 and 10 μg/mL) in 0.2μm filtered NSW. A control group (only NSW) was also included. The experiments were run at least in triplicate and carried out at least six times. Flasks (50 mL) were kept in a thermostatic chamber at 25 ± 0.5 °C in dark conditions without providing food. After 48 h, 15 nauplii were randomly sampled from each concentration (5 nauplii per flask in triplicate) six times, and total body length of 90 individuals were determined using a Leica MZ6 stereo microscope fitted with a digital camera (Leica hz6). Images were analyzed with the open-source software ImageJ Version 1.52a (National Institutes of Health, USA). Simultaneously, development stage was determined according to Amat (1985). For each organism, the length from the cephalic region to the telson (excluding antennule and caudal rami) was measured, according to the procedure reported in Bergami et al.

(2017). Before, nauplii were anaesthetized with a few drops of a solution of distilled water saturated with chloroform. The remaining nauplii (≈ 195) from each individual flask were collected, rinsed with mQW and stored at -80ºC for biochemical analyses or in RNA later at -20ºC for gene analysis.

The long-term assay was performed according to Varó et al. (2015) and Comeche et al. (2017), with some modifications. Briefly, 100 newly hatched nauplii (Instar I) were transferred to 150 mL Erlenmeyer flask filled with 100 mL of experimental solution during the first 7 days, and with 150 mL until 14 days. The final experimental solutions were prepared with filtered NSW (0.20 μm) containing the fresh microalgae *Tetraselmis suecica* with a density of $12x10^4$ cell/mL for the first 3 days, $15x10^4$ cell/mL from day 3 to day 9 and 20 $x10^4$ cell/mL from day 9 to day14. First, three concentrations of PS-NH_2 (0.1, 1 and 3 μg/mL) plus a control group were tested, but due to high mortality rate observed at 3 μg/mL, for biomarkers and gene expression analyses, additional experiments were run with only two concentrations of PS-NH_2 (0.1 and 1 μg/mL).

The organisms were kept under controlled condition of temperature (25 ± 0.5 ºC) with moderate aeration and photoperiod 16:8 h light:dark in a thermostatic chamber. Complete medium renewal was performed four times in 14 days to maintain the exposure conditions and to minimize NP aggregation. Four independent replicates were set for each concentration, and the experiment was repeated at least 3 times. Data on growth were calculated using total body length which was measured as described above. The number of individuals measured from each PS-NH_2 concentration tested at each time varied between 134-205 according to survival. Survival was recorded at 3, 6, 9 and 14 days of exposure. Also, developmental stage was determined in randomly sampled individuals (between 20-30) from each concentration at 3 and 14 days of exposure. Furthermore, feeding behavior was evaluated at 7 and 14 days of exposure.

To evaluate the filtration and ingestion rates upon exposure to PS-NH_2, 24 individuals (3 individuals per 4 replicates, run twice) from each concentration were randomly selected and placed in 6-well plates (1 individual/mL) filled with 6 mL filtered NSW (0.2 μm) during 1 h without food. The individuals were then transferred to 12-well plates (1 individual/mL) filled with 3 mL of microalgae *T. suecica* at a density of 12 $x10^4$ cell/mL during 3 h. Blank samples in triplicate for each treatment were prepared

with the same volume of microalgae density without individuals. During the feeding experiment the multi-well plates were placed in a thermostatic chamber at 25 ± 0.5 ºC in total darkness. At the end of the experiment, 1mL from each well was collected and the remaining algal cells counted using a Neubauer chamber, and individuals discarded. The average of filtering (F) and ingestion rates (I) were calculated according to the formulae given by Gauld (1951).

Filtering rate (µL/ind/h):

$$F = \frac{V}{n} x \frac{LnCo - LnCt}{t} - A; \qquad A = \frac{LnCo - LnCt}{t}$$

Where: V= medium volume (µL); n=number of individuals; Co= initial cell concentration (cell/µL); Ct=final cell concentration (cell/µL); t= time of feeding (hours); A= change in cell concentration in blank during the time of feeding experiment.

Ingestion rate (n cell/ind/h):

I= F x $\sqrt{Co - Ct}$

At the end of exposure time (14 days) the remaining individuals were sampled, rinsed in distilled water and stored at -80ºC for biochemical and molecular analyses.

2.4 Biomarkers

2.4.1 Sample preparation

After short-term assay, the biomarkers were determined using pools of 48 h old nauplii (≈ 195 individuals), except for lipid peroxidation in which 5 pools were randomly combined (≈ 975 individuals). After long-term assays, pools of 40 individuals were sampled for enzymatic and lipid peroxidation assays, and pools of 15 individuals for HSP70 and gene expression analyses. Samples for enzymatic and lipid peroxidation assays were manually homogenized using a microcentrifuge tube pestle in 200 µL ice-cold buffer phosphate (100 mM phosphate buffer pH 7.4 with 150 mM KCl; 1mM EDTA and 0,1% Triton-X), following the procedure described in Comeche et al. (2017). In brief, homogenates were sonicated (see 2.1 for details) twice during 2 min separated by a period of freezing of 30 min, and the volumes adjusted up to 600 µL for nauplii or 1200 µL for juveniles (14 days old) with buffer.

Then, the homogenates were centrifuged at 10,000 g at 4 °C for 15 min, and the supernatants were stored in aliquots at −80 °C until analyses.

For HSP70 quantification, samples were manually homogenized using a microcentrifuge tube pestle in 1:10 (w:v) ice-cold calcium-magnesium free saline buffer (20 mMHepes, 500 mMNaCl, 12.5 mMKCl (pH=7.3)), freshly complemented with 1mM dithiothreitol (DTT), 1mM phenymethysulfonyfluoride (PMSF), Igepal (1%) and 1% protease inhibitor cocktail (Complete-Mini, EDTA-free ROCHE). Samples were centrifuged at 10000 g (4°C) for 20 min and the supernatants were stored in aliquots at −80 °C until analyses.

2.4.2 Biomarker assays

Cholinesterases (ChE) activity was determined according to the methodology described in Varó et al., (2015), using acetylthiocholine (ASCh) as substrate. Sample volume used was 100 µL in a total volume of 300 µL. Absorbance was read at 415 nm (ε_{415} = 13.6 mM^{-1} cm^{-1}) during 30 min. ChE characterization in *A. franciscana* was performed using specific inhibitors of ChE, such as eserine sulphate, tetraisopropylpyrophosphoramide (iso-OMPA) and 1,5-bis-(4-allyldimethyl-ammoniumphenyl)-pentan-3-one dibromide (BW284C51) to determine the predominant form of ChE (Varó et al., 2002).

Carboxylesterase (CbE) activity was determined following Mastropaolo and Yourno method (1981) adapted to microplate as described in Comeche et al. (2017) using p-nitrophenyl acetate (pNPA) as substrate. Sample volume used was 25 µL in a total volume of 225 µL. The absorbance was immediately read at 405 nm (ε_{405} = 23.4 $mM^{-1}cm^{-1}$) during 3 min. GST activity was determined according to Habig et al. (1974) adapted to microplate as described in Comeche et al. (2017) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Sample volume used was 50 µL in a total volume of 250 µL. The absorbance was read at 340 nm (ε_{340} = 9.6 mM^{-1} cm^{-1}) during 15 min. CAT activity was determined by measuring the decrease of H_2O_2 concentration according to the method of Aebi (1974), adapted by Comeche et al. (2017) for brine shrimp. The reaction mixture containing phosphate buffer (100 mM, pH 6.5) and H_2O_2 (50 mM) as substrate, was freshly added and the absorbance immediately read at 340 nm (ε_{340} = 40 M^{-1} cm^{-1}) in a UV microplate during 1 min. Sample volume used was 50 µL in a total volume of 250 µL.

Lipid peroxidation was determined by measuring the formation of thiobarbituric reactive substances (TBARS) and quantified as malondialdehyde (MDA) equivalents. TBARS were measured after incubation of homogenate at 95 °C with acetic acid (pH 3.4) and thiobarbituric acid (Ohkawa et al. 1979), following the procedure described in Varó et al (2007) with some modifications as reported below. In brief, 50 µL of sample were treated with 8.1% dodecyl sulfate with butylated hydroxytoluene (BHT, 0.005%), 20% trichloroacetic acid (pH 3.5) and 0.8% thiobarbituric acid and heated for 60 min in boiling water. After cooling, the mixture was made up to 0.5 mL with distilled water, n-butanol and pyridine (15:1, v/v) added, and shaken vigorously before centrifugation at 10,000 g for 5 min. The supernatant (organic layer) was then collected and its fluorescence measured at 530 nm/550 nm EX/EM. MDA concentrations were derived from a standard curve using 1,1,3,3-tetraethoxypropane as standard.

All biomarkers were run in triplicate. Absorbance and fluorescence were read in a TECAN Ultra Evolution microplate at 25ºC, using the kinetic mode when needed. The results are expressed as nmol/min/ mg of protein (ChE, CbE and GST) or µmol/min/ mg of protein (CAT). Lipid peroxidation is expressed as nmol TBARS/mg of protein.

HSP70 levels were determined following the procedure described in Solé et al (2015) with some modifications. Protein samples (20 µg) were separated using Bio-Rad Mini-Protean TGX Precast gels (4-20% resolving gel) in a Mini-Protean® Tetra cell system (Bio-Rad) and transferred onto PVDF membranes (0.2 µm, Trans-Blot® Turbo™ Mini PVDF Transfer Packs) in a Trans-Blot® TurboTM Blotting System (Bio-Rad). Membranes were incubated with blocking buffer (5% non-fat dry milk in 1x TBS) for 1 h at room temperature. The immunodetection was performed using HSP70 mouse monoclonal antibody (Sigma H5147) diluted 1:5000 in 3% non-fat dry milk in 1x TBS, and anti-mouse IgG secondary antibody conjugated with peroxidase (Sigma A4416) diluted 1:25000 in 3% non-fat dry milk in 1x TBS. Blots were developed using Amersham ECL PRIME kit as recommend by manufacturer and visualized on Amsterdam TM imager 600.The intensity of the HSP70 bands in the blots was quantified by densitometry using the ImageQuant TL Software (Amersham. 8.1). The density of each band was normalized to the density of the HSP70 band of a commercial standard (Sigma, H9776) in each blot (Varó et al. 2007), and HSP70 levels are expressed as arbitrary units HSP70 per ng of protein.

Total soluble protein contents of samples for ChE, CbE, GST and LP were determined using Bradford Bio-Rad Protein Assay, adapted to microplate and the absorbance was read at 595 nm. For HSP70 samples, protein content was determined by using Lowry Bio Rad DC-Protein Assay kit, and the absorbance was read at 700 nm. BSA (bovine serum albumin) was used as standard.

2.5 RNA extraction, cDNA synthesis and real time q-PCR

Gene expression analysis was performed following the procedure reported in Vannuccini et al. (2015). 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 (2 mg) was transcribed to cDNA using qScriptTM XLT One-Step RT-qPCR ToughMix® (Quanta Biosciences) according to manufacturer's protocol. Selected genes *cstb, clap, tcp, hsp70* and *hsp26* were investigated through quantitative Real Time PCR (RT q-PCR), using *glyceraldehyde 3-phosphatase dehydrogenase* (*gapdh*) as housekeeping gene. Primers of these genes are listed in Supplementary Table 1S. RT q-PCR was performed using a Stratagene Mx 3000P thermal cycler. 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 5 μL of PerfeCta SYBR Green FastMix, Low ROX 2x (Quanta BiosciencesTM). The cycling conditions were: 95 °C for 30 s for initial denaturation, followed by 40 PCR cycles 5 s at 95 °C, 15 s at specific melting T °C for each couple of primers (Supplementary Table 1S), 10 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 reactions. Real-time efficiency of primer set (E) was determined for each gene from the slopes given by MxProTM QPCR software (Stratagen, USA), apply the equation $E = 10^{(-1/slope)}$.

2.6 Statistical analysis

Data were checked for normality and homogeneity of variances by Shapiro-Wilk and Levene tests respectively. Survival data in long-term assay was analyzed using

Kaplan-Meier curves, and Wilcoxon test used for pairwise comparisons of survival curves. The effect of the two main factors (PS-NH_2 and developmental stage) on growth was tested by two-ways analysis of variance (2ways-ANOVA), followed by HDS Tukey *post hoc* test.

The effect of PS-NH_2 exposure on biochemical biomarkers was assessed by one-way ANOVA, with Brown-Forsythe test for heterogeneous variances when needed, followed by HDS Tukey *post hoc* test for homogeneous variances or Games-Howell *post hoc* test for heterogeneous variances. A t-test was used to compare the normal range of activity in non-exposed 0 and 48 h old nauplii of. For all data, $p \leq 0.05$ value was considered statistically significant (95% confidence).

Data obtained from gene expression analysis are expressed as mean ± standard deviation (SD) and were analyzed by one-way ANOVA, using Tukey *post hoc* and $p \leq 0.05$ as significant cut-off.

Statistical analyses on biochemical biomarkers were carried out using IBM SPSS Statistic, while the ones on gene expression were performed using Graphpad Prism 5.

3. Results

3.1 Behaviour of PS-NH_2 in natural sea water (NSW)

DLS results from physico-chemical characterization of PS-NH_2 in mQW and NSW media are summarized in Table 1. PS-NH_2 resulted well dispersed in mQW over time (0 – 72 h), with a Z-Average of 52.8 ± 0.9 nm close to the nominal size of 50 nm and PDI of 0.202±0.044 (Supplementary Fig. 1S). The optimal dispersion and stability in mQW (Supplementary Fig. 1S) were confirmed also after 48 h (mean Z-Average 56.3 nm and PDI 0.145) and 72 h (mean Z-Average 56.9 nm and PDI 0.152) of incubation under controlled experimental conditions (Table 1).

The ζ-potential measured by electrophoretic mobility technique confirmed the positive surface charge (+38.5 ± 2.5 mV) of the particles suspended in in mQW, in agreement with Bergami et al. (2017) and Manfra et al. (2017).

On the contrary, PS-NH_2 slightly aggregated in NSW at 0 h (Supplementary Fig. 1S), as shown by a Z-Average value of 148.5 ± 20.6 nm, broader PDI (> 0.350) and higher instability in this medium, with a lower absolute ζ-potential value of +13.9 ± 2.7 mV.

NP aggregation in this medium increased significantly over time (Table 1) and large sedimentation aggregates in NSW were observed after 48 h and 72 h of incubation (Supplementary Fig. 2S A) at the same exposure conditions of *Artemia*, and further confirmed by TEM (Supplementary Fig. 2S C) compared to mQW (Supplementary Fig. 2S B).

3.2 Survival, growth and feeding behaviour

High mortality was recorded (>70%) in nauplii exposed at the highest PS-NH_2 (10 μg/mL) upon short-term exposure (48 h). A significant concentration-dependent reduction in total body length ($p \leq 0.05$) was observed at 1 and 10 μg/mL but no differences were found at the lowest concentration (0.1 μg/mL) (Fig. 1A). Nauplii exposed to 10 μg/mL PS-NH_2 became surrounded by a clump of exuviates and NP-aggregates (Supplementary Fig.3S). This was not found in those exposed to 1 μg/mL PS-NH_2. Some of the nauplii exposed both to 1 and 10 μg/mL remained in instar II-III (Fig. 2A) instead of progressing to instar III-IV, as did control and 0.1 μg/mL PS-NH_2 exposed nauplii.

Survival was significantly affected by long-term exposure (14 days) to 0.1, 1 and 3 μg/mL PS-NH_2 (Long Rank, $p \leq 0.05$) and significant differences were observed between exposed and control groups (Pairwise comparisons $p \leq 0.05$). It is interesting to know, that only 18.4 ± 5.1 % of survival was found at the highest PS-NH_2 concentration tested (3 μg/mL) (Fig.3). Total body length increased ($p \leq 0.05$) with development (until 14 days) (Fig.1B), but was unaffected by PS-NH_2 exposure. At morphological level, developmental alterations were detected after three days of exposure to 1 and 3 μg/mL PS-NH_2 (Fig. 2B). However, after 14 days, in all experimental groups individuals in juvenile stage were observed. Feeding behavior, determined as ingestion and filtration rates, was not affected by PS-NH_2 (0.1 and 1 μg/mL PS-NH_2) after 7 and 14 days of exposure (Supplementary Fig.6S).

3.3 Biomarkers

The high mortality of nauplii after 48 h exposure to 10 μg/mL PS-NH_2 did not allow to collect enough individuals for running biomarker assays. Therefore, all enzymatic activities were measured in pools of nauplii exposed to 0.1 and 1 μg/mL.

No concentration-dependent decrease in ChE activity was observed in nauplii after 48

h exposure to PS-NH_2 (Fig. 4). A significant increase compared to controls was observed ($p \leq 0.05$) in nauplii exposed to 0.1 μg/mL, while a decrease was found in those exposed to 1 μg/mL compared to control. CbE activity, on the other hand, resulted significantly inhibited ($p \leq 0.05$) in a concentration-dependent manner compared to controls (Fig. 4). Based on the data obtained using specific inhibitors of ChE (eserine), AChE (BW284C5) and BChE (Iso-OMPA) enzymes, a significant ($p \leq 0.05$) contribution of AChE enzyme on the measured ChE *vs* ASCh activity was determined confirming its role (Supplementary Fig. 4S) in hydrolyzing ASCh substrate in brine shrimp tissues.

In terms of oxidative response and detoxification, both GST and CAT activities resulted significantly reduced ($p \leq 0.05$) upon PS-NH_2 exposure even though no differences were observed between 0.1 and 1 μg/mL in GST, while CAT activity was only significantly affected at 1 μg/mL ($p \leq 0.05$). Likewise, HSP70 protein levels resulted significantly higher ($p \leq 0.05$) only in nauplii exposed to 1 μg/mLPS-NH_2.

Enzymatic activities were also measured in control nauplii (only NSW) at Instar I stage (newly hatched) and at Instar II and III stages (after 48h) (Supplementary Fig.5S). The results showed a significant increase in all enzymatic activities and LP levels with developmental stage (age), while no changes were observed for HSP70 protein levels.

In long-term exposure, only individuals exposed to 0.1 and 1 μg/mL PS-NH_2 were used due to high mortality (81.6 ± 5.1 %) observed at the highest concentration tested (3 μg/mL). A significant decrease ($p \leq 0.05$) in ChE, CbE, GST and CAT activities was found in individuals exposed at 1 μg/mL, while HSP70 levels significantly increased ($p \leq 0.05$). However, a dose-response of LP ($p \leq 0.05$) was observed in all exposed groups (0.1 and 1 μg/mL).

3.4 Gene expression

A dose-dependent modulation of selected genes (*cstb, clap, tcp, hsp26* and *hsp70*) was observed after 48h and 14 days of PS-NH_2 exposure and they were found significant at 1 μg/mL ($p \leq 0.05$) compared to the control groups (Fig. 5).

Comparing the two times of exposure, a general stronger induction was observed in 48 h nauplii with respect to 14 days juveniles, excluding *tcp* gene, which was significantly up-regulated in 14 days brine shrimps in control and 0.1 μg/mL PS-NH_2

451 groups to 1.18 and 1.25-fold respectively ($p \leq 0.05$). In particular, *cstb* and *clap* genes
452 involved in the development resulted on average 1.05 and 0.85-fold more induced
453 respectively in 48 h nauplii exposed to 1 μg/mL PS-NH_2 compared to 14 days
454 juveniles ($p \leq 0.05$ for both *cstb* and *clap*). Moreover, *hsp26* and *hsp70* were found
455 3.09 and 5.59-fold more induced respectively in 48h brine shrimps compared to 14
456 days juveniles ($p \leq 0.05$ and $p \leq 0.0001$ for *hsp26* and *hsp70* respectively) exposed to
457 1 μg/mL PS-NH_2.
458
459 **4. Discussion**
460 The physico-chemical characterization of PS-NH_2 in NSW at 0 h is in agreement with
461 our previous findings obtained under similar conditions (T = 25°C, comparable
462 salinity and pH values) (Bergami et al., 2016, 2017). DLS and TEM analyses over
463 time (48 – 72 h) indicate an important increase in aggregation and sedimentation of
464 micro-scale NP aggregates, lowering their bioavailable to *A. franciscana*. However,
465 during both 48 h and 14 days exposures *A. franciscana* individuals were found to
466 swim continuously in the culture medium with NP aggregates, probably generating
467 smaller aggregates of PS-NH_2 as shown by TEM images in NSW (aggregates of ~ 500
468 nm).
469 The "multiple molting effect" in nauplii of *A. franciscana* found after short-term
470 exposure at 10 μg/mL PS-NH_2 observed in the present work had been already
471 reported for 50 and 100 μg/mL PS-NH_2 (Bergami et al., 2016). We have also
472 recorded a decrease in size together with a delay in the developmental stage for 1 and
473 10 μg/mL PS-NH_2 groups. However, development alterations in *Artemia* may not be
474 exclusive of PS-NH_2, as Bhubaneshwar et al. (2018) also reported morphological
475 changes in *Artemia* nauplii upon waterborne exposure to titanium dioxide NPs (TiO_2
476 NPs).
477 Regarding the long-term experiments, no differences in growth were found after 3, 6,
478 9 and 14 days of exposure in any of the concentrations tested. This is coincident with
479 the results found by Bergami et al. (2017) at 14 days. Interestingly enough, the
480 morphology of individuals was affected after 3 days of exposure to 1 and 3 μg/mL
481 PS-NH_2 (see Fig 2B), under the form of alterations in the budding thoracopods. After
482 14 days of exposure, the surviving individuals in all treatments reached the juvenile
483 stage, characterized by the presences of 11 pairs of thoracopods, short antennas which

have lost their long setae, undergoing sexual differentiation (Amat, 1985). The long-term exposure to 1 and 3 μg/mL PS-NH_2 could have selected only the most resistant organisms in such a way that those exhibiting morphological and/or developmental alterations are non-viable. In fact, the survival of 43.5 ± 15.1 % and 18.4 ± 5.1 % found in groups exposed to 1 and 3 μg/mL PS-NH_2 respectively, supports our previous findings of a LC_{50} (14 days) of 0.83 μg/mL, with 100% mortality at 5 μg/mL PS-NH_2 (Bergami et al., 2017). It is important to remark that despite the PS-NH_2 agglomeration in NSW, each medium renewal assured a pulse of bioavailable NP for *Artemia* directly from water, that is responsible for the high mortality rate in the long-term exposure. Also, PS-NH_2 taken in with the food, due to physical adsorption of PS-NH_2 positively charged on microalgae, or that comes in contact with the external body surface of *Artemia*, can cause the mortality found (Bergami et al.,2017).

As far as the mechanisms of toxicity for microplastics and NPs (including nanoplastics) in *Artemia* is concerned, accumulation in the digestive tract of *Artemia* has been acknowledged as one of the main causes of mortality and toxicity. Although the excretion is limited by the formation of NPs aggregates into the gut, it can be improved by the presence of food (Ates et al., 2013, Bergami et al., 2016, 2017, Bhuvaneshwari et al., 2018, Madhav et al., 2017).

The lack of effect on feeding behaviour in *Artemia* during long-term exposure to PS-NH_2 (0.1 and 1 μg/mL) suggests that in the present study PS-NH_2 agglomeration in NSW can reduce their uptake, as well as the formation of the aggregates in the digestive tract due to the presence of food during long-term exposures reported by Ates et al., (2013) and Bergami et al., (2016, 2017).

Regarding biochemical responses, both short- and long-term exposures to PS-NH_2 led to significant variations in all biomarkers, such as ChE, GST and CAT activities, except for lipid peroxidation (LP), which was not affected in short-term exposure.

The decrease of ChE activity at 1 μg/mL PS-NH_2 found both after short and long-term exposures indicates that PS-NH_2 caused neurotoxicity and may contribute to the high mortality found at 14 days. The fact that nauplii after a short-term exposure to the same concentration of NP exhibited a decrease in ChE activity with not such extensive mortality may be related to the high tolerance of nauplii of the genus *Artemia* to ChE inhibition. No lethal effects were seen in *A. salina* and *A parthenogenetica* after 80% of inhibition (Varó et al., 2002). ChE characterization

through the use of inhibitors showed that true Acetylcholinesterase (AChE) is the main ChE form present in nauplii *A. franciscana*, in contrast with the presence of both AChE and BChE in *A. salina* and *A. parthenogenetica* (Varó et al., 2002). This finding indicates that important differences in response to PS NH_2 exposure among species of *Artemia* genus may exist.

With respect to the opposite response found in nauplii after 48 hours exposure to 0.1 and 1 mg/mL PS-NH_2, the existence of non-monotonic doses-response curve as described by Fagin (2012) may be the explanation. Cholinesterase activity (ChE) responses to metallic and carbon-based nanomaterials have been found in different *Artemia* species (Gambardella et al., 2014, Mesarič et al., 2015). Whether the enzyme activity response to PS-NH_2 is due to the direct physicochemical effect of PS-NP, or any of its components, on the enzyme, or the result of complex interactions with the cellular and physiological mechanisms, as demonstrated for other kinds of NP (Gambardella et al., 2014, Wang et al., 2009), is beyond the present study.

As far as we know, no previous studies have reported the effect of PS-NH_2 or other nanomaterials on CbE activity on invertebrates, including *Artemia*. Although detoxification is the most well-known function for CbEs, this group of enzymes are also responsible for the metabolism of endogenous substances like arthropod juvenile hormones, in whose catabolism specific carboxylesterases have been found to be involved (Goodman and Granger, 2005; Tao et al., 2017). Methyl farnesoate (MF) is the juvenile hormone in crustaceans (Chang and Kaufman, 2005). Our results show inhibition of CbE activity in nauplii exposed to PS-NH_2 for 48 h in a concentration-dependent manner. The role of carboxylases in the catabolism of juvenile hormones in insects (Goodman and Granger, 2005), and the cause-effect relation between high levels of juvenile hormones or alike with an abnormal development in insects are known (Willis et al., 1987). Taken all together, it seems plausible that the "multiple molting" effect of *Artemia* nauplii, found for the first time in a previous study (Bergami et al., 2016) and also observed in the present study, as well as the existence of abnormally developed individuals after 48 and 72 hours of PS-NH_2 exposure, may be a case of endocrine disruption mediated by CbE inhibition and the consequent deregulation of MF levels. Some of the resulting developmental and morphological alterations may be lethal and thereby the reason for the high mortality observed later. The lack of concomitance between inhibition of carboxylesterases after 14 days of exposure with morphological abnormalities, may be consequence of an age-related

551 sensitivity to MF.
552 The increase in lipid peroxidation, one of the main effects of ROS presence in the
553 cells, after 14 days of exposure at both concentrations tested, indicates oxidative stress
554 as one of the mechanisms causing PS-NH_2 long term toxicity. However, this effect
555 was not detected in short-term exposure. When analyzing responses in antioxidant
556 enzymes (CAT and GST) a decline of activity was observed in both short and long-
557 term exposures at the highest concentration, and only at 14 days at the low
558 concentration. The inhibition of CAT activity observed, after short- and long-term PS-
559 NH_2 exposures, pointing out that oxidative stress response measured as LP, is
560 probably an accumulative effect caused by an increase of H_2O_2 due to the decrease in
561 antioxidant enzyme activities (CAT and GST). Oxidative stress has been described on
562 the toxicity studies of nanomaterials in species of the genus *Artemia* (Ates et al.,
563 2013, Bhuvaneshwari et al., 2018, Gambardella et al., 2014, Mesarič et al., 2015).
564 According to our results this effect is easier to be detected after long term exposure
565 experiments than in short term ones.
566 HSPs constitute an additional cell mechanism to face stressful conditions, playing a
567 crucial role in maintaining protein homeostasis under stress conditions. The protective
568 function of HSP70 against oxidative stress, apoptosis and inflammatory processes in
569 response to various kinds of environmental and physiological stressors is well
570 documented (Barauh et al., 2012, Ikwegbue et al., 2018, Roberts et al., 2010). In the
571 present study, the increase in HSP70 levels at the highest PS-NH_2 concentration, both
572 after 48 h and 14 days could be part of the cellular response against oxidative stress
573 induced by PS-NH_2 (Roberst et al., 2010), together with antioxidant enzymes and LP.
574 A further confirmation of this defense mechanism is given by the gene expression
575 profile of both *hsp70* and *hsp26*, which was significantly up-regulated in brine
576 shrimps exposed to 1 μg/mL PS-NH_2 at both 48 h and 14 days. HSP70 is also known
577 to be involved in cytoprotection, regulating protein folding/unfolding and degradation
578 as a stress consequence, preventing the cell to undergo apoptosis (Gething and
579 Sambrook 1992; Feder and Hofmann,1999; Zhou et al., 2010). The small chaperone
580 HSP26 also plays an important role in protein protection from irreversible
581 denaturation against oxidative stress, moving from the nucleus and stabilizing nuclear
582 matrix proteins (Cheng et al., 2009).
583 These results at the molecular level are in line with HSP70 function and support the
584 hypothesis of a clear stress response induced by PS-NH_2 after 48 h, in agreement with

recent findings on other model organisms exposed to micro- (Imhof et al., 2017) and nanoplastics (Pinsino et al., 2017; Brandts et al., 2018).

After long-term exposure, *hsp26* did not show any further increase, while a significant up-regulation of *hsp70* was still observed at the lowest PS-NH_2 concentration after 14 days compared to the control. Likewise, *tcp* gene showed a significant up-regulation still after 14 days of exposure to PS-NH_2 at 0.1 μg/mL with respect to short-term exposure in which the expression of this gene was similar to controls. The chaperonin-containing TCP is known as cell cycle regulator, having a role in the folding of newly translated proteins (Chen et al., 2009). Our results confirm the specific protective role of *hsp70* in cellular defense upon prolonged exposure to PS-NH_2 and support the involvement of HSPs and *tcp* in *Artemia* long-term responses to this stressor.

Regarding the other genes, a similar trend in *clap* and *cstb* modulation after short- and long-term exposure was observed. Cathepsin L-like protease is involved in key processes related to *Artemia* development, in particular in molting, while cystatin B inhibitor is known to regulate the activity of this protease (Chen et al., 2009). The results obtained in the present study are in agreement with previous findings on 48 h exposed *Artemia* nauplii (Bergami et al., 2017) and confirm that *clap* and *cstb* genes are modulated by PS-NH_2 inducing multiple molting over long-term exposures.

5. Conclusions

Our findings demonstrate that brine shrimp *A. franciscana,* a model organism of aquatic zooplankton, is sensitive to short- and long-term exposure to cationic amino-modified PS-NH_2 (50 nm), but some responses are dependent on the timing of exposure. On one hand, short-term exposure to PS-NH_2 causes a decrease in nauplii growth and affect the development in a concentration-dependent manner. On the other hand, in long-term experiments, survival, but not growth and feeding behavior of juveniles, get impaired. Oxidative stress is detected after short-term exposure under the form of a decrease in the activity of antioxidant enzymes, but it is fully evident in the long-term as lipid peroxidation, suggesting an accumulative effect. The decrease observed in ChE activity suggests a possible neurotoxic action of PS-NH_2. Also, Carboxylesterase (CbE) inhibition by PS-NH_2, described for the first time in this

study, anticipates potential effects in biotransformation of exogenous and endogenous compounds, being the crustacean juvenile hormone methyl farnesoate (MF), that regulates development and molting, one candidate. A further confirmation of the ability of PS-NH_2 to affect the expression of genes involved in cell protection, development and molting is reported under short- and long-term exposure conditions which are more likely to occur in natural exposure scenarios and in high polluted regions. Overall, our results reveal that low PS-NH_2 concentrations induce physiological, biochemical and molecular (changes in gene expression) alterations in *Artemia,* and point to their potential risk for this model organism. This supports the general concern about nanoplastics occurrences in aquatic environments, and their ability to represent an ecological threat for aquatic zooplanktonic species.

Acknowledgments

This work was financed by Generalitat Valenciana (GV-2014/085-Prometeo-II), and talian Antarctic Research Program (PNRA) contract number: PNRA 16_0075 NANOPANTA: Nano-Polymers in the Antarctic mariNe environmenT and biotA. PS NP characterization by DLS analysis was conducted at facilities available at the Department of Biotechnologies, Chemistry and Pharmacy of the University of Siena (Italy).

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Table 1. Physico-chemical characterization of PS-NH_2 in milli-Q water (mQW, T = 25 ± 0.5°C) and natural sea water (NSW, 0.20 µm filtered, T = 25°C, salinity 38.29‰, pH 8.25) by DLS, showing Z-average (nm) and polydispersity index (PDI) after 0, 48 and 72 h of incubation in the media. Data are referred to NP concentration of 50 µg/mL and values reported as average ± standard deviation (SD) of 3 independent measurements.

	Time	Z-average (nm)	PDI
mQW	0 h	52.8 ± 0.9	0.202 ± 0.04
	48 h	56.3 ± 2.0	0.145 ± 0.01
	72 h	56.9 ± 1	0.152 ± 0.05
NSW	0 h	148.5 ± 0.6	0.355 ± 0.03
	48 h	> 3500	> 0.500
	72 h	> 5000	> 0.400

Figure
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Fig.1

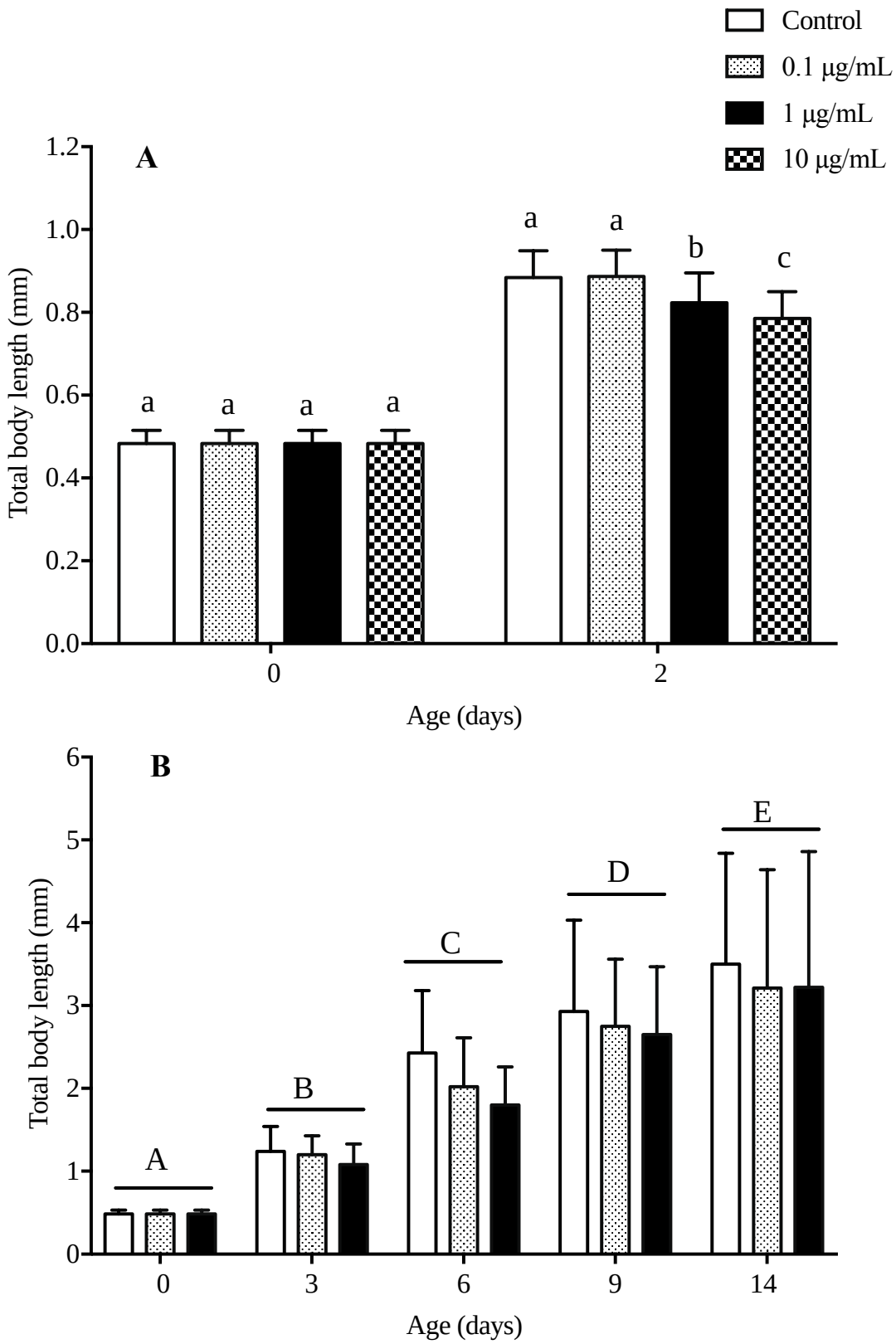


Fig. 2 A. Short-term exposure

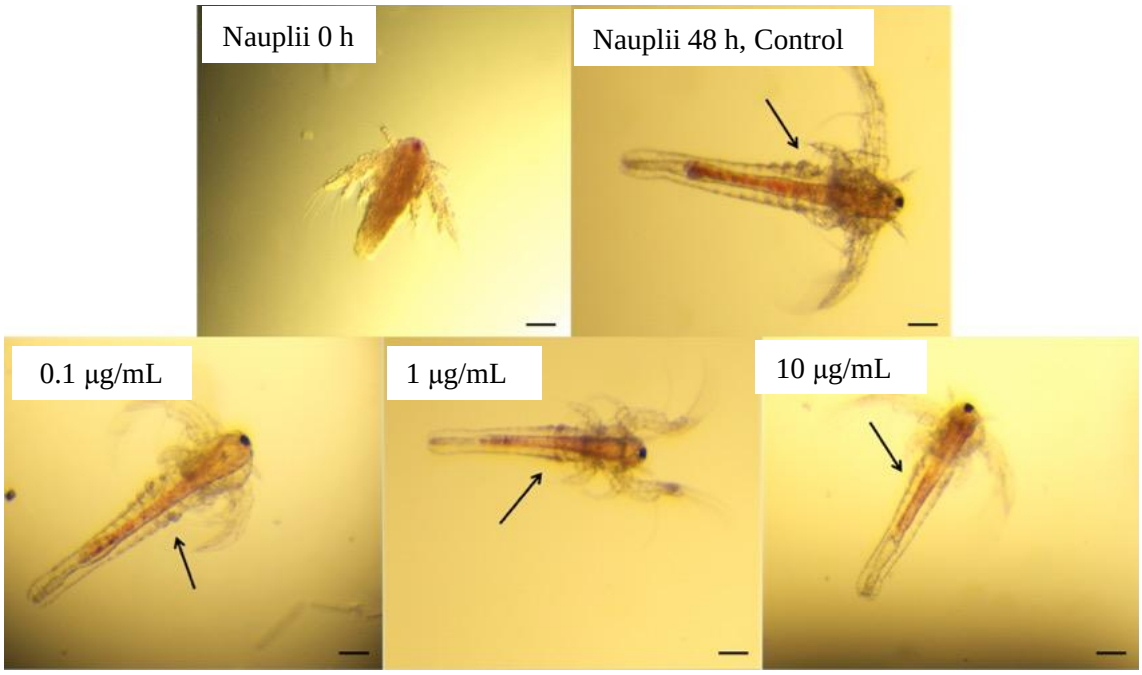
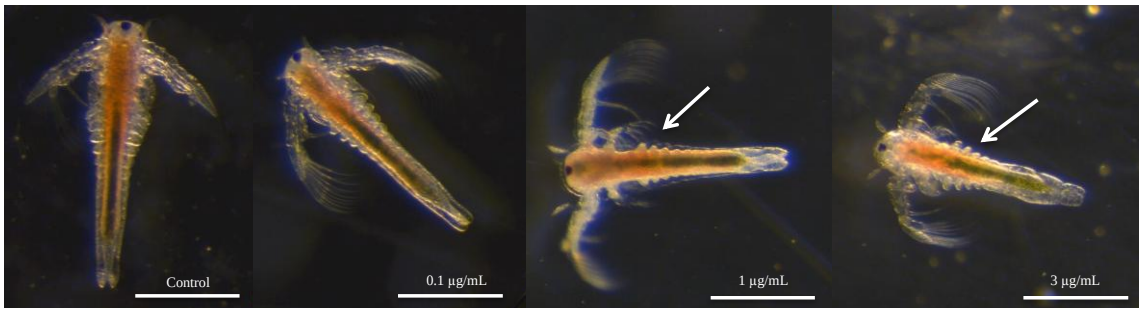
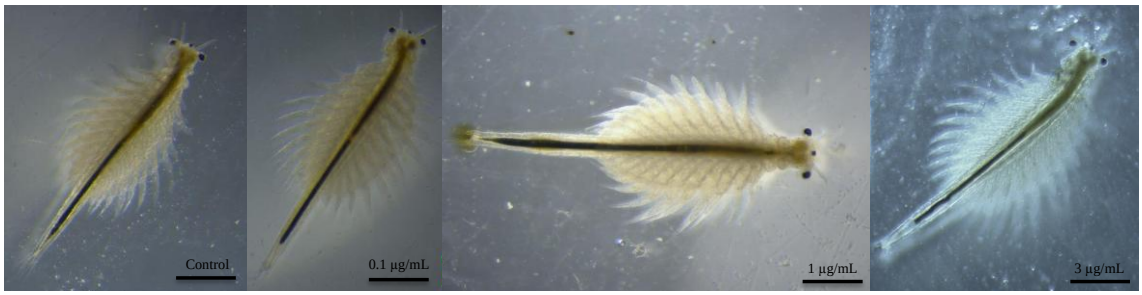


Fig. 2 B. Long-term exposure



Metanauplii 3 days



Juveniles 14 days

Fig. 3

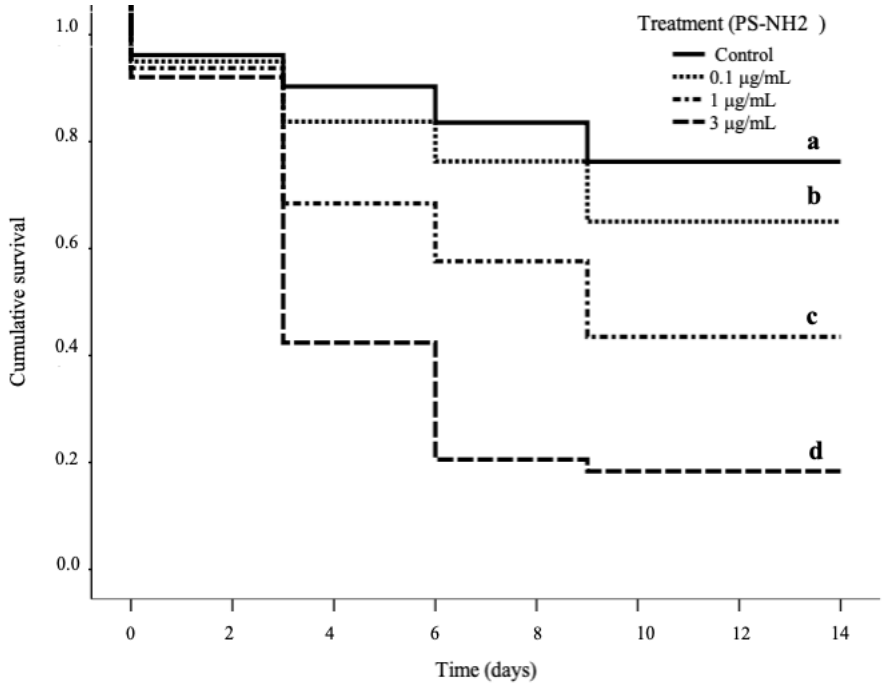

Treatment (PS-NH2)
Control
0.1 µg/mL
1 µg/mL
3 µg/mL
a
b
c
d
Cumulative survival
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
Time (days)

Fig. 4

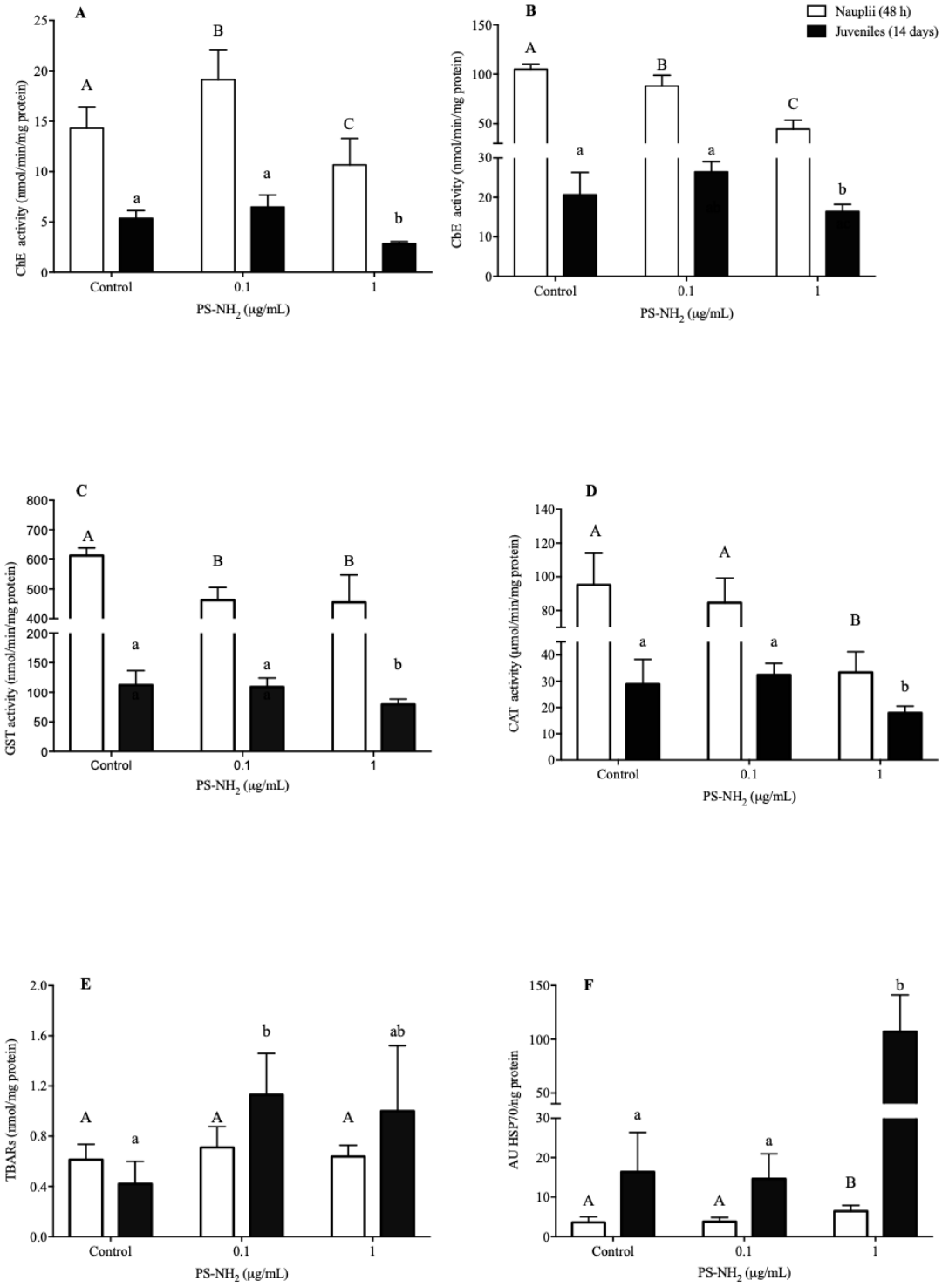
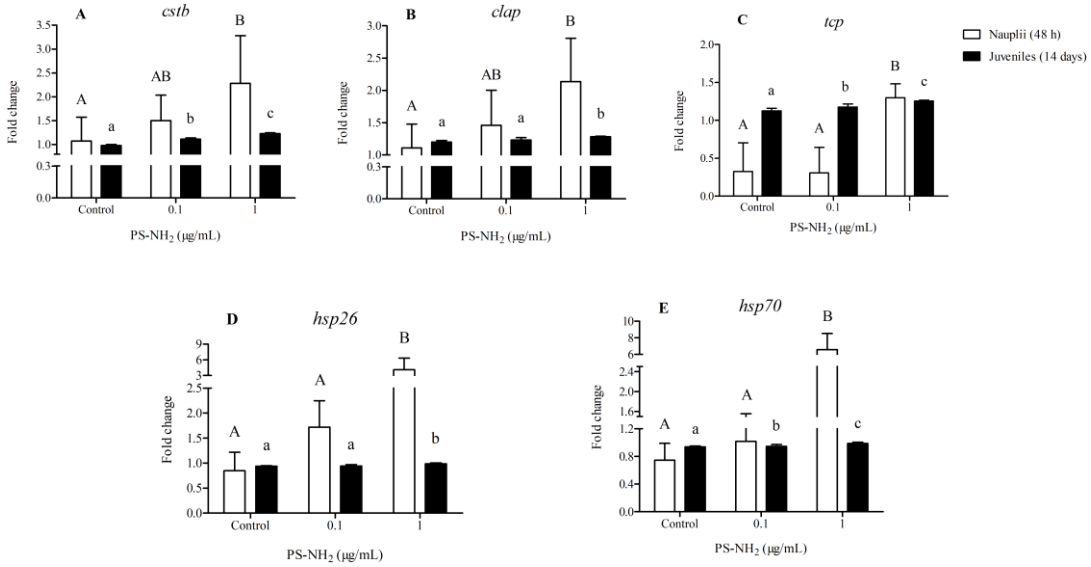

A
ChE activity (nmol/min/mg protein)
PS-NH2 (µg/mL)
Control
0.1
1
B
CbE activity (nmol/min/mg protein)
Nauplii (48 h)
Juveniles (14 days)
C
GST activity (nmol/min/mg protein)
D
CAT activity (µmol/min/mg protein)
E
TBARs (nmol/mg protein)
F
AU HSP70/ng protein

Fig. 5


A
cstb
B
clap
C
tcp
Nauplii (48 h)
Juveniles (14 days)
D
hsp26
E
hsp70
Fold change
Control
0.1
1
PS-NH2 (µg/mL)

Legends

Fig. 1. Effect of short (48 h) and long-term (14 d) exposure to different suspensions of PS-NH_2 on brine shrimp *A. franciscana* growth (total body length, mm). Lower-case letters denote significant differences between treatments and capital letters denote significant differences with development (age) (ANOVA and Tukey *post hoc* test, $p \leq 0.05$). Values are expressed as mean ± standard deviation (SD). Short-term exposure n = 90 per treatment and long-term exposure n = 134-205 per treatment.

Fig. 2. Morphological changes in development in brine shrimp *A. franciscana* after short, (A) and long-term (B) exposure to different suspensions of PS-NH_2. Arrows indicate morphologic differences in development. Scale bar: 100 μm (A) and 500 and 1000 μm for metanauplii 3 days and juveniles 14 days old, respectively (B).

Fig. 3. Cumulative survival (%) of brine shrimp *A. franciscana* after long-term exposure (14 d) to different suspensions of PS-NH_2 (Control, 0.1, 1 and 3 μg/mL). Different letters indicate significant difference between treatments (Pairwise comparisons, $p \leq 0.05$).

Fig. 4. Cholinesterase (ChE, A), carboxylesterase (CbE, B), glutathione-S-transferase (GST, C), catalase (CAT, D) enzyme activities, lipid peroxidation (TBARs, E) and heat shock proteins levels (HSP70, F) in brine shrimp of *A. franciscana* after short (48 h) and long-term exposure (14 d, days) to PS-NH_2 (Control, 0.1 and 1 μg/mL). Values are expressed as mean ± standard deviation (SD). Short-term exposure n = 3-6 and long-term exposure n = 4-8 per treatment. Capital letters denote significant differences in nauplii and lower-case letters denote significant differences in juveniles (ANOVA and Tukey *post hoc* test, $p \leq 0.05$).

Fig. 5. Genes expression of *cstb* (A), *clap* (B), *tcp* (C), *hsp70* (D), *hsp26* (E) in brine shrimp of *A. franciscana* after short (48 h) and long-term exposure (14 d, days) to PS-NH_2 (Control, 0.1 and 1 μg/mL). Results are shown as mean ± standard deviation (SD). Short-term exposure n = 3-6 and long-term exposure n = 3. Capital letters denote significant differences in nauplii and lower-case letters denote significant differences in juveniles (ANOVA and Bonferroni *post hoc* test, $p \leq 0.05$).

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