Impact of cationic polystyrene nanoparticles (PS-NH2) on early embryo development of Mytilus galloprovincialis: Effects on shell formation

Impact of cationic polystyrene nanoparticles (PS-NH_2) on early embryo development of *Mytilus galloprovincialis*: Effects on shell formation

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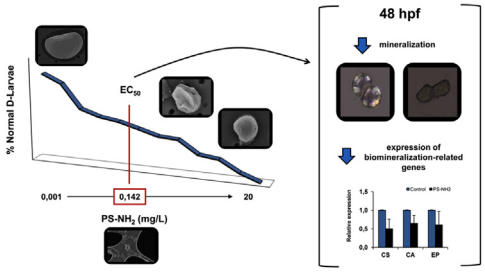
HIGHLIGHTS

- Effects of amino modified nano polystyrene PS NH_2 on *Mytilus* early development.
- At 0.150 mg/L ($\cong EC_{50}$) malformed D veligers, decreased mineralization and shell size.
- Transcription of 12 genes was evalu ated by RTqPCR at 24 and 48 hpf.
- At 48 hpf, selective downregulation of genes involved in shell formation.
- At higher concentrations, develop mental arrest/high embryotoxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

The potential release of nanoparticles (NPs) into aquatic environments represents a growing concern for their possible impact on aquatic organisms. In this light, exposure studies during early life stages, which can be highly sensitive to environmental perturbations, would greatly help identifying potential adverse effects of NPs. Although in the marine bivalve *Mytilus* spp. the effects of different types of NPs have been widely investigated, little is known on the effects of NPs on the developing embryo. In *M. galloprovincialis*, emerging contaminants were shown to affect gene expression profiles during early embryo development (from trocophorae 24 hpf to D veligers 48 hpf). In this work, the effects of amino modified polystyrene NPs (PS NH_2) on mussel embryos were investigated. PS NH_2 affected the devel opment of normal D shaped larvae at 48 hpf ($EC_{50} = 0.142$ mg/L). Higher concentrations (5 20 mg/L) resulted in high embryotoxicity/ developmental arrest. At concentrations $\cong EC_{50}$, PS NH_2 affected shell formation, as shown by optical and polarized light microscopy. In these conditions, transcription of 12 genes involved in different biological processes were evaluated. PS NH_2 induced dysregulation of tran- scription of genes involved in early shell formation (Chitin synthase, Carbonic anhydrase, Extrapallial Protein) at both 24 and 48 hpf. Decreased mRNA levels for ABC transporter p glycoprotein ABCB and Lysozyme were also observed at 48 hpf. SEM observations confirmed developmental toxicity at higher

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concentrations (5 mg/L). These data underline the sensitivity of *Mytilus* early embryos to PS NH_2 and support the hypothesis that calcifying larvae of marine species are particularly vulnerable to abiotic stressors, including exposure to selected types of NPs.

1. Introduction

The continuous production and usage of nanoparticles (NPs) will inevitably lead to their environmental release in substantial amounts into water compartments, with potential adverse effects for aquatic organisms (Baker et al., 2014; Corsi et al., 2014). In this light, the utilization of early life stage toxicity tests, involving exposure during the most sensitive stages of the organism to environmental stress, would greatly help in the identification of those NPs that represent a major threat to aquatic species (Paterson et al., 2011; Zhang et al., 2012; Gambardella et al., 2015). This also applies to studies on the biological impact of NPs on marine invertebrates where, complimentary to the use of adult specimens, embryos have emerged as valid tools for studies on developmental perturbations induced by different types of NPs (Corsi et al., 2014; Canesi and Corsi, 2016). Echinoderms have been so far the most studied taxonomic group of marine invertebrates in assessing NP developmental toxicity (reviewed in Canesi and Corsi, 2016). Several types of NPs (metal based NPs and metal oxides, carbon based NPs, nanopolymers etc.) resulted in different degrees of embryotoxicity in different sea urchin species at concentrations of μg mg/L (Canesi and Corsi, 2016). Although these studies demonstrated that in the sea urchin model embryo development can represent a significant target for different types of NPs, in a concentration range of μg/L low mg/L, information on the underlying molecular mechanisms is still extremely scarce. Only a few data are available in bivalve molluscs: in the oyster *Crassostrea virginica* C_{60} fullerene affected embryonic development from concentrations as low as 10 μg/L (Ringwood et al., 2009, 2010). In contrast, in the mussel *Mytilus galloprovincialis*, metal oxide NPs (n Fe_2O_3 and n TiO_2) were ineffective unless at high mg/L concentrations (Kadar et al., 2010; Libralato et al., 2013; Balbi et al., 2014).

Nanoplastics represent an emerging type of NPs of environmental concern. The continuous increase of plastic wastes and debris in the aquatic environment, including estuarine and coastal areas, is increasing the worldwide attention on the possible impact of micro and nano plastics on marine biota (Moore, 2008; Mattsson et al., 2015; Lambert and Wagner, 2016). Polystyrene (PS) is among the most largely used plastics worldwide, accounting for 24% of the macroplastics in the estuarine habitat, and it can be found in the oceans and in marine organisms as micro debris (Browne et al., 2008; Moore, 2008; Andrady, 2011; Plastics Europe, 2013). These studies also suggested that microplastic will subsequently degrade into nano sized plastic particles. The formation of nanoplastics during the degradation of polystyrene has been clearly demon strated (Lambert and Wagner, 2016).

Recent data showed that polystyrene NPs affect embryo devel opment in marine invertebrates: in the sea urchin *Paracentrotus lividus,* amino modified polystyrene NPs (PS NH_2) caused severe developmental defects and changes in gene expression at both 24 and 48 hpf (Della Torre et al., 2014). In brine shrimp larvae (*Artemia franciscana*) PS NPs were shown to affect food uptake (feeding), behavior (motility) and physiology (multiple molting) (Bergami et al., 2016). PS NH_2 has been shown to affect the immune func tion in *M. galloprovincialis* (Canesi et al., 2015, 2016); however, no information is available on the possible impact of nanoplastics on embryo development of bivalve species.

In *M. galloprovincialis*, changes in gene transcription occurring during early development, from fertilized eggs to the trocophora (24 hpf) and to the first D veliger (48 hpf) have been recently investigated. The results underlined the molecular mechanisms involved in the early critical stages, such as the formation of the first shelled embryo, and the adverse effect of estrogenic compounds on transcription of genes related to neuroendocrine signaling and biomineralization (Balbi et al., 2016).

In this work, the possibility that amino modified polystyrene NPs (PS NH_2) may affect early embryo development in *M. galloprovincialis* was investigated by a morphological and transcriptomic approach. Fertilized eggs were exposed to different concentrations of PS NH_2 in a wide concentration range (from 0.001 to 20 mg/L) and EC_{50} values were determined in the 48 h embryotoxicity test (Fabbri et al., 2014). The effects of selected concentrations of PS NH_2 on larval morphology at 48 hpf, were evaluated in terms of shell size and degree of mineralization by optical and polarized light microscopy, respectively. Moreover, the effects of PS NH_2, at concentrations close to the EC_{50}, on gene expression were investigated at both 24 and 48 hpf in comparison to eggs. Transcriptional profiles of 12 selected genes related to known biological functions and whose expression is regulated across early embryo development in physiological conditions (Balbi et al., 2016) were evaluated. These include genes involved in biomineralization, neuroendocrine signaling, immune response, antioxidant defense, biotransformation, autophagy and apoptosis. The effects of higher concentrations of PS NH_2 on shell morphology at 48 hpf were also evaluated by and scanning electron microscopy (SEM).

2. Methods

2.1. PS NH_2 characterization

Primary characterization of unlabelled 50 nm amino polystyrene NPs (PS NH_2), purchased from Bangs Laboratories, was performed as previously described (Della Torre et al., 2014; Canesi et al., 2015, 2016; Bergami et al., 2016). PS NH_2 suspensions (50 μg/mL) were prepared in artificial sea water (ASW) (pH 8, salinity 36‰; ASTM, 2004) and filtered with 0.22 μm membrane. Suspensions were quickly vortexed prior to use but not sonicated. Size (Z average and polydispersity index, PDI) and zeta potential (ζ potential, mV) were determined by Dynamic Light Scattering (Malvern instruments), using a Zetasizer Nano Series software, version 7.02 (Particular Sciences, UK). Measurements were performed in triplicate, each containing 11 runs of 10 s for determining Z average, 20 runs for the ζ potential.

PS NH_2 suspensions in ASW (25 μg/mL) were also pelleted by centrifugation and resuspended in MilliQ water, in order to eliminate the excess NaCl, and resuspended in 1 mL of MilliQ water. After vortexing, two drops of the suspension were placed on a lacey carbon holder and left to dry in air without coating. Samples were observed by field emission scanning electron microscopy (FESEM) on a ZeissSUPRA40VP scanning electron microscope operating at

20 kV and by transmission electron microscopy (TEM) (Tecnai G2 Spirit BioTWIN Philips, Eindhoven) The Netherlands). The results on characterization of PS NH_2 suspensions are summarized in Fig. S1.

2.2. Mussels and gamete collection

Sexually mature mussels (*M. galloprovincialis* Lam.), purchased from an aquaculture farm in the Ligurian Sea (La Spezia, Italy) be tween November and March, were transferred to the laboratory and acclimatized in static tanks containing aerated artificial sea water (ASTM, 2004), pH 7.9–8.1, 36 ppt salinity (1 L/animal), at 16 ± 1 °C. Mussels were utilized within 2 days for gamete collection. When mussels beginning to spontaneously spawn were observed, each individual was immediately placed in a 250 mL beaker containing 200 mL of aerated ASW until complete gamete emission. After spawning, mussels were removed from beakers and sperms and eggs were sieved through 50 μm and 100 μm meshes, respectively, to remove impurities. Egg quality (shape, size) and sperm motility were checked using an inverted microscope. For each experiment, eggs and sperm from two individuals were selected and counted to give a single pairing. Eggs were fertilized with an egg:sperm ratio 1:10 in polystyrene 96 microwell plates (Costar, Corning Incorporate, NY, USA). After 30 min fertilization success (n. fertilized eggs/n. total eggs × 100) was verified by microscopical observation (>85%).

2.3. Embryotoxicity test

The 48 h embryotoxicity assay (ASTM, 2004) was carried out in 96 microwell plates as described by Fabbri et al. (2014). Aliquots of 20 μL of 10x suspensions of PS NH_2 (obtained from a 20 g/L stock suspension in MilliQ water), suitably diluted in filter sterilized ASW, were added to fertilized eggs in each microwell to reach the nominal final concentrations (0.001 0.01 0.05 0.1 0.25 0.5 1 2.5 5 10 20 mg/L) in a 200 μL volume. At each dilution step, all sus pensions were immediately vortexed prior to use. Microplates were gently stirred for 1 min, and then incubated at 18 ± 1 °C for 48 h, with a 16 h:8 h light:dark photoperiod. All the following procedures were carried out following ASTM 2004. At the end of the incubation time, samples were fixed with buffered formalin (4%). All larvae in each well were examined by optical and/or phase contrast micro scopy using an inverted Olympus IX53 microscope (Olympus, Milano, Italy) at 40×, equipped with a CCD UC30 camera and a digital image acquisition software (cellSens Entry). Observations were carried out by an operator blind to the experimental condi tions. A larva was considered normal when the shell was D shaped (straight hinge) and the mantle did not protrude out of the shell, and malformed if had not reached the stage typical for 48 hpf (trocophore or earlier stages) or when some developmental defects were observed (concave, malformed or damaged shell, protruding mantle). The recorded endpoint was the percentage of normal D larvae (D veligers) in each well respect to the total, including malformed larvae and pre D stages. The acceptability of test results was based on controls for a percentage of normal D shell stage larvae >75% (ASTM, 2004).

In a separate set of experiments, the embryotoxicity test was also carried out by exposing either fertilized eggs or embryos developed at 24 hpf to a single concentration of PS NH_2 (0.150 mg/L).

All other experiments were carried out adding PS NH_2 to fertilized eggs, unless otherwise indicated.

Light microscopy images of D veligers at 48 h in different experimental conditions were also analyzed for shell length (the anterior posterior dimension of the shell parallel to the hinge line) and height (the dorsal ventral dimension perpendicular to the hinge) (Kurihara et al., 2007, 2009).

2.4. Polarized light microscopy and scanning electron microscopy (SEM)

For observations by polarized light microscopy, control embryos and embryos treated with 0.150 mg/L PS NH_2 grown in 96 microwell plates at 48 hpf were collected by filtration on 0.20 μm filters (about 500 embryos/sample). Each sample was washed four times with deionized water to remove excess salts, and dried at 60 °C for 30 min. Observations (40x) were carried out on glass slides by a polarized light microscope (OLYMPUS BX 41). Images were acquired by an Olympus Color view II and digitalized by the Olympus Color view II Bund Cell B.

For SEM observations, control embryos and embryos treated with 5 mg/L PS NH_2 at 48 hpf were fixed in 3% glutaraldehyde in ASW. After fixation, samples from 6 microwells were pooled, placed onto Whatman 22 μm filters, dehydrated in an ascending series of ethanol washes (50%–80%–90%–100%) and air dried. Then sam ples were sputter coated with gold, and observed at 20 kV with a Vega3 Tescan scanning electron microscope. All procedures were carried out as previously described (Balbi et al., 2016).

2.5. RNA extraction and qRT PCR analysis

Unfertilized eggs (about 24,000 eggs/ml) obtained from at least 6 female individuals were collected by centrifugation at 400 × g for 10 min at 4 °C, and the resulting pellet was frozen in liquid nitrogen. Eggs were fertilized with an egg:sperm ratio 1:10 in polystyrene 6 well plates and a final 8 mL volume. After 30 min, fertilization success (n. fertilized eggs/n. total eggs x 100) was verified by microscopical observation (>85%). At 30 min pf, PS NH_2 was added to fertilized eggs in each well from 20 g/L concentrated stock so lutions prepared in MilliQ water, immediately vortexed and suit ably diluted to reach the final nominal concentration of 0.150 mg/L. Concentration was chosen on the basis of the dose response curve obtained in the *Mytilus* 48 h embryotoxicity test (present study). Control wells (negative controls) contained only ASW. Four repli cates for each experimental condition were made.

At 24 and 48 hpf larvae were collected by a nylon mesh (20 μm pore filter) and washed with ASW. Three wells for each condition were pooled in order to obtain approximately 7000 embryos/replicate. The larval suspension was centrifuged at 800 × g for 10 min at 4 °C. Larval pellets and unfertilized eggs were lysed in 1 mL of the TRI Reagent (Sigma Aldrich, Milan, Italy) and total RNA was extracted following manufacturer's instructions. RNA concentration and quality were verified using the Qubit RNA assay (Thermo Fisher, Milan, Italy) and electrophoresis using a 1.5% agarose gel under denaturing conditions. First strand cDNA for each sample was synthesized from 1 μg total RNA (Balbi et al., 2014). Gene transcription was evaluated in 4 independent RNA samples.

Primers pairs employed for qRT PCR analysis were as reported in previous studies or were designed with Primer Express (Thermo Fisher, Milan, Italy) using nucleotide sequences retrieved from the GeneBank database (https://www.ncbi.nlm.nih.gov/genbank/) for *Mytilus galloprovincialis* (Table S1). qPCR reactions were performed in triplicate in a final volume of 15 μL containing 7.5 μL iTaq universal master mix with ROX (BioRad Laboratories, Milan, Italy), 5 μL diluted cDNA, and 0.3 μM specific primers (Table S1). A control lacking cDNA template (no template) was included in the qPCR analysis to determine the specificity of target cDNA amplification. Amplifications were performed in a StepOne real time PCR system apparatus (Thermo Fisher, Milan, Italy) using a standard "fast mode" thermal protocol. For each target mRNA, melting curves

were utilized to verify the specificity of the amplified products and the absence of artifacts. HEL and EF α1 were utilized as the best performing combination of reference gene products (EF1/HEL) for data normalization (Balbi et al., 2016). Calculations of relative expression of target mRNAs was performed by a comparative C_T method (Schmittgen and Livak, 2008) using the StepOne software tool (Thermo Fisher, Milan, Italy). Data were reported as relative expression (fold change or log2 transformed fold changes according to the data ranges) with respect to unfertilized eggs (basal gene expression across larval development) or to control samples within each life stage (PS NH_2 treatment).

2.6. Data analysis

Embryotoxicity test data were expressed as means ± SDs of 4 experiments carried out in 6 replicate wells. Data on gene transcription were obtained from 4 independent RNA samples and data expressed as means ± SDs. Statistical differences were evaluated with respect to controls ($P \leq 0.05$, Mann Whitney *U* test). Deviations from parametric test assumptions were verified through the Shapiro Wilk's test (Normality) and the Bartlett's test (equal variance). The EC_{50} was defined as the concentration of chemical causing 50% reduction in the embryogenesis success, and 95% confidence intervals (CI) were calculated by PRISM 5 software (GraphPad Prism 5 software package, GraphPad Inc).

Gene transcription data were also submitted to permutation multivariate analysis of variance (PERMANOVA) using PRIMER v6 (Anderson et al., 2008). Log transformed fold changes were used to calculate similarity matrices (Euclidean distance, 999 permutations). Factors considered were "developmental stage" and "PS NH_2" treatment. Pseudo F values in the PERMANOVA main tests were evaluated in terms of significance (Anderson et al., 2008). When the main test revealed statistical differences ($P < 0.05$), permutation t tests through PERMANOVA pairwise comparisons were carried out (Euclidean distance matrix, 999 permutations). Distance based redundancy linear modeling (DISTLM) followed by a redundancy analysis (dbRDA) in PRIMER was also performed to examine the relationship between the multivariate dataset (i.e. the suite of biological endpoints assayed and their variations) and the predictor variables (life stage and PS NH_2 treatment). Numerical metric for life stage progression was indicated by the post fertilization time (0 h: unfertilized egg; 24 h: trocophorae; 48 h: D veliger). Treatment was indicated by the nominal concentrations of PS NH_2 exposure (150 μg/L). DISTLM used the BEST selection procedure and adjusted R^2 selection criteria.

3. Results

3.1. Effects of PS NH_2 on embryo development

Fertilized eggs were exposed to different concentrations (from 0.001 to 20 mg/L) of PS NH_2 in 96 microwell plates, and the percentage of normal D larvae was evaluated after 48 hpf. The results, reported in Fig. 1, show that PS NH_2 induced a dose dependent decrease in normal larval development, with an EC_{50} value of 0.142 mg/L (0.09178–0.2345 mg/L) (Fig. 1A). The effect was significant from 0.01 mg/L (34%; $P \leq 0.01$) and was dramatic (80%) from concentrations ≥ 2.5 mg/L. As shown in Fig. 1B, the effect of exposure to PS NH_2 was biphasic: PS NH_2 at lower concentrations (from 0.001 to 1 mg/L) mainly induced malformations of the D veligers. At higher concentrations (from 2.5 mg/L to 10 mg/L), a delay in development was observed, with a progressive increase in the presence of embryos at the pre veliger stage and a stable proportion of embryos still at the trocophora stage. At the highest concentrations tested (20 mg/L) PS NH_2 completely inhibited the formation of the D shaped veliger, with about 90% of the larvae withheld at the trocophora stage. Almost identical results were observed in experiments carried out in both 2015/16 and 2016/17 (not shown).

In a separate set of experiments, the 48 h embryotoxicity test was carried out adding a single concentration of PS NH_2 close to the obtained EC_{50} values (0.150 mg/L) to either fertilized eggs or embryos at 24 hpf, when all embryos were developed into the trocophora stage. Although the time of exposure did not significantly affect the percentage of normal D veligers (i.e. the endpoint of the embryotoxicity test), a higher percentage of immature embryos (pre veligers and trocophorae) was observed when PS NH_2 were added to trocophorae with respect to fertilized eggs.

All subsequent experiments were carried out adding PS NH_2 to fertilized eggs as in the standard embryotoxicity assay. Represen tative light microscopy images of control embryos and embryos exposed to different concentrations of PS NH_2 (0.150–1–2.5–5 mg/L) at 48 hpf, showing both malformed and immature embryos, are reported in Fig. S2 (A D). When the size of D veligers was

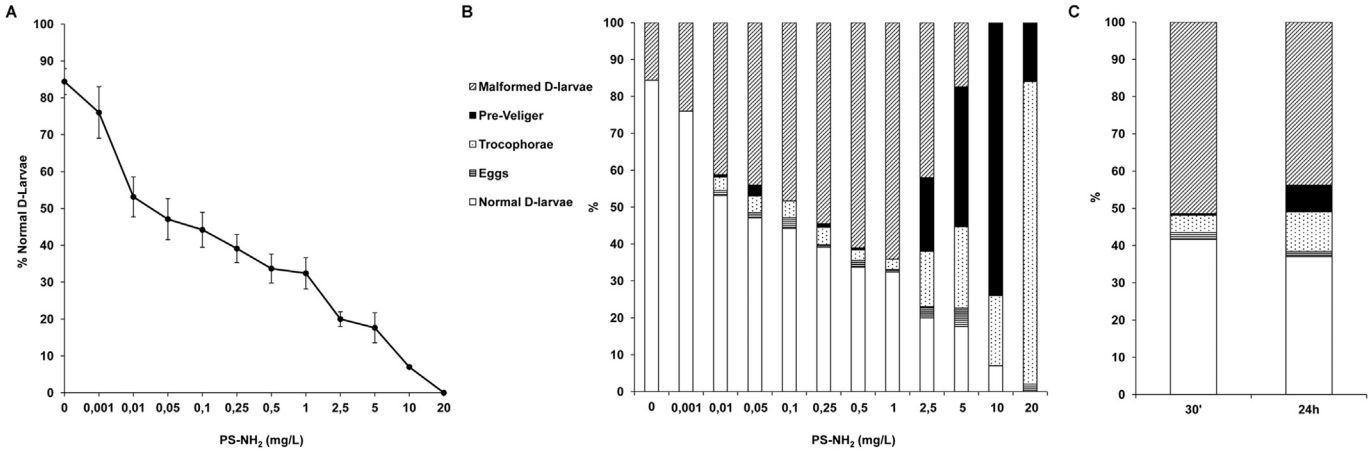

Fig. 1. Effects of different concentrations of PS-NH_2 (0.001-0.01-0.05-0.1-0.25-0.5-1-2.5-5-10-20 mg/L) on *M. galloprovincialis* normal larval development in the 48 h embryotoxicity assay. A and B show the results obtained by exposure of fertilized eggs to PS-NH_2. A) Percentage of normal D-shaped larvae with respect to controls. B) Percentage of normal D-veliger (dark grey), malformed D-veliger (light grey) and pre-veligers (white) and trocophorae in each experimental condition. C) same as in B, but showing the effects of addition of a single concentration of PS-NH_2 (0.150 mg/L) to either fertilized eggs or at 24 hpf. Data represent the mean ± SD of 4 experiments carried out in 96-multiwell plates (6 replicate wells for each sample).

evaluated, a small but significant decrease in shell length (20 30%) was recorded in PS NH_2 exposed samples, irrespective of the exposure concentration, whereas shell height was unaffected (Fig. S2E).

Fertilized eggs were exposed to PS NH_2 (at a concentration of 0.150 mg/L, close to the EC_{50} values obtained at 48 hpf) and D veligers were observed by polarized light microscopy, to evaluate the degree of shell mineralization, based on the observed birefringence due to the mineral phase (Weiss et al., 2002; Kurihara et al., 2007, 2009). Representative images reported in Fig. 2 show that control D veligers exhibited a rather weak but evident birefrangence over most of the shell area, in particular along the hinge and the margins of the valvae, indicating partial shell mineralization (Fig. 2A and B). In contrast, in malformed D veligers from PS NH_2 treated samples birefrangence was almost absent (Fig. 2C and D).

3.2. Effects of PS NH2 on gene transcription

The effects of exposure of fertilized eggs to PS NH_2 (0.150 mg/L) on gene transcription was evaluated at different times pf (24 and 48 h). Transcription of 12 selected genes related to neuroendocrine signaling (serotonin receptor, 5 HTR), antioxidant defense (catalase CAT, superoxide dismutase SOD), biotransformation (glutathione transferase GST, ABC transporter p glycoprotein ABCB), biomineralization (extrapallial protein, EP, carbonic anhydrase, CA), autophagy, growth and metabolism (serine/threonine protein ki nase mTor), apoptosis (p53), immune response (Toll like receptor, TLR i), was evaluated. All these genes, except for GST, were previously shown to be up regulated across different stages (from eggs to 24 h and 48 hpf) under normal physiological conditions (Balbi et al., 2016). In addition, transcription of chitin synthetase CS and Lysozyme LYSO, genes involved in shell formation and immune response/intracellular digestion, respectively, was evaluated. The results of basal expression of CS and LYSO in untreated embryos are shown in Fig. S3. Data, reported as log2 transformed relative expressions with respect to unfertilized eggs, indicate a slight down regulation of CS at 24 hpf, followed by a strong up regulation at 48 hpf. Levels of mRNA for Lysozyme were extremely low at 24 hpf in comparison to eggs; however, a large increase in transcription was observed from 24 to 48 hpf.

The effects of PS NH_2 exposure on gene expression are shown in Fig. 3. As shown in Fig. 3A, PS NH_2 induced up regulation of CS, CA and EP at 24 hpf, in particular of CS (1.7 fold increase with respect to controls; $P \leq 0.05$). In contrast, a general down regulation of all genes was observed at 48 hpf (about 40%). The effect was significant for both CS and CA (35% and 50%, respectively; $P \leq 0.05$). A similar trend was observed in transcription of the ABCB gene, with significant up regulation at 24 hpf (+46%; $P \leq 0.05$) and down regulation at 48 hpf (44%; $P \leq 0.05$). PS NH_2 also induced a significant decrease in mRNA levels for Lysozyme at both times pf (36 and 46%, respectively, $P \leq 0.05$). No effects were observed in transcription of all the other genes at 24 and 48 hpf. On the whole, results from PERMANOVA and permutation *t* test analyses demonstrated that the effects of PS NH_2 on gene transcription were statistically significant only in the D veliger stage ($P < 0.05$; Table S2). Indeed, distance based linear model (DISTLM) analysis revealed that though expression profiles were strongly dependent on embryo development (explaining about 91% total variation, in agreement with Balbi et al., 2016), PS NH_2 treatment accounted for about 6.3% of total variation, which mostly explained the observed changes in transcript expressions at 48 hpf (Fig. 3B). PERMANOVA analysis also showed a significant interaction between the two factors "developmental stage" and "PS NH_2" treatment ($P < 0.05$; Table S2).

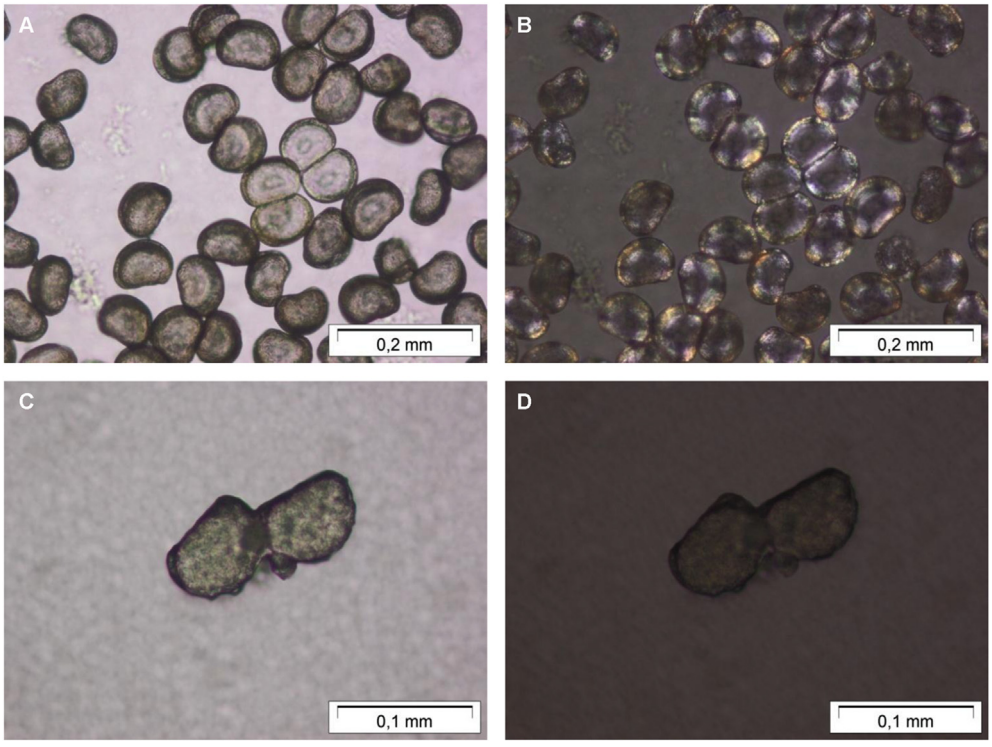

Fig. 2. Effects of exposure of fertilized eggs to PS-NH_2 (0.150 mg/L) on *Mytilus* embryos at 48 hpf observed by polarized light microscopy. Left panels: light microscopy. Right panels: polarized light microscopy. A, B) control embryos, showing weak but evident shell birefringence consistent with a fraction of crystalline shell material (Prodissoconch I); C, D) embryos exposed to 0.150 mg/L PS-NH_2, where no birefringence could be observed, indicating the prevalence of amorphous calcium carbonate (ACC) in the shell.

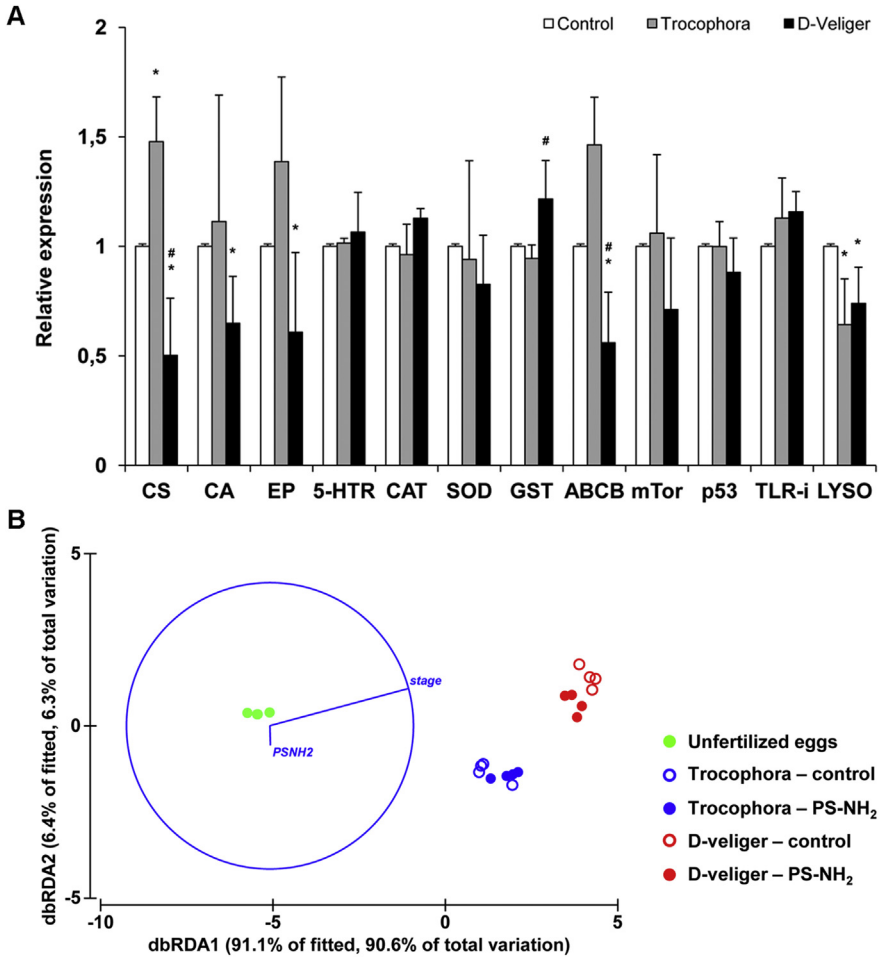

Fig. 3. Effects of exposure of fertilized eggs to PS-NH_2 (0.150 mg/L) on gene transcription in *Mytilus* embryos at 24 and 48 hpf. A) Relative expression of CS (chitin synthetase), CA (carbonic anhydrase), EP (Extrapallial Protein), 5-HTR (5-hydroxyl triptamine receptor), CAT (catalase), Superoxide dismutase (SOD), GST (glutathione transferase), ABCB (ABC transporter p-glycoprotein), mTor (mammalian target of rapamycin), p53, TLR i (Toll-like receptor i isoform), LYSO (Lysozyme). Data are reported as mean ± SD of the relative expression with respect to untreated samples with in each life stage (N 4). *P < 0.05, (Mann-Whitney *U* test). Further statistical differences were evaluated between trocophorae and D-veligers (#P < 0.05). B) Distance-based redundancy (DISTLM) modeling with distance-based redundancy analysis (dbRDA) to explore the amount of the variation in gene transcription to be attributed to PS-NH_2 treatment (150 µg/L) of *Mytilus* embryos at different developmental stages (Euclidean Distance resemblance matrix, 999 permutations).

3.3. Scanning electron microscopy (SEM)

Control samples and samples exposed to higher concentrations of PS NH_2 (5 mg/L) at 48 hpf were also observed by SEM as previously described (Balbi et al., 2016), and the results are reported in Fig. 4. Fig. 4A shows a representative image of a normal D veliger, characterized by a shell with straight hinge, symmetric valvae, and uniform surface. In PS NH_2 treated samples, the results confirm the presence of embryos still at the trocophora stage (Fig. 4B); shelled embryos were represented by malformed D veligers and preveligers, characterized by externalized velum (arrows) and showing thin valvae with irregular surfaces (Fig. 4C–E). Small preveligers with shells made of thin cracked lamellar structures were also observed (Fig. 4E–F). Malformed embryos covered in small PS NH_2 agglomerates (Fig. 4G, I). These latter had with a size ranging from 200 to 500 nm (Fig. 4H, L). These agglomerates often showed a rough surface, suggesting that they were surrounded by organic material (Fig. 4H).

4. Discussion

The results demonstrate that PS NH_2 significantly affect *M. galloprovincialis* early development in a wide concentration range, with an EC_{50} of 0.142 mg/L. These data indicate a much higher susceptibility of *Mytilus* embryos to PS NH_2 in comparison with different types of nano oxides (Kadar et al., 2010; Libralato et al., 2013; Balbi et al., 2014). Moreover, the same PS NH_2 have been previously shown to induce developmental toxicity in the sea urchin *Paracentrotus lividus*, with EC_{50} values of 3.82 mg/L and 2.61 mg/L at 24 and 48 hpf, respectively. In particular, at 48 hpf, PS NH_2 caused various larval alterations as incomplete or absent skeletal rods, fractured ectoderm, reduced length of the arms and high percentage of blocked embryos (Della Torre et al., 2014). The EC_{50} obtained in the 48 h mussel embryotoxicity test was one order of magnitude lower than that calculated for the sea urchin, further underlying the sensitivity of *Mytilus* embryos to PS NH_2. The effects of NPs on marine invertebrates are influenced by their behavior in high ionic strength media (Canesi and Corsi, 2016). Characterization of PS NH_2 in sea water suspensions indicated that these particles retain a positive surface charge, with ζ potential of +14 mV in ASW(Della Torre et al., 2014; Canesi et al., 2015, 2016; Bergami et al., 2016). In contrast, negatively charged PS COOH did not show sea urchin embryotoxicity up to concentrations of 50 mg/L (Della Torre et al., 2014). Similarly, metal oxide NPs such as n TiO_2, that also show negative ζ potentials in ASW (Doyle et al., 2014) did not affect mussel embryo development (Libralato et al., 2013). Since

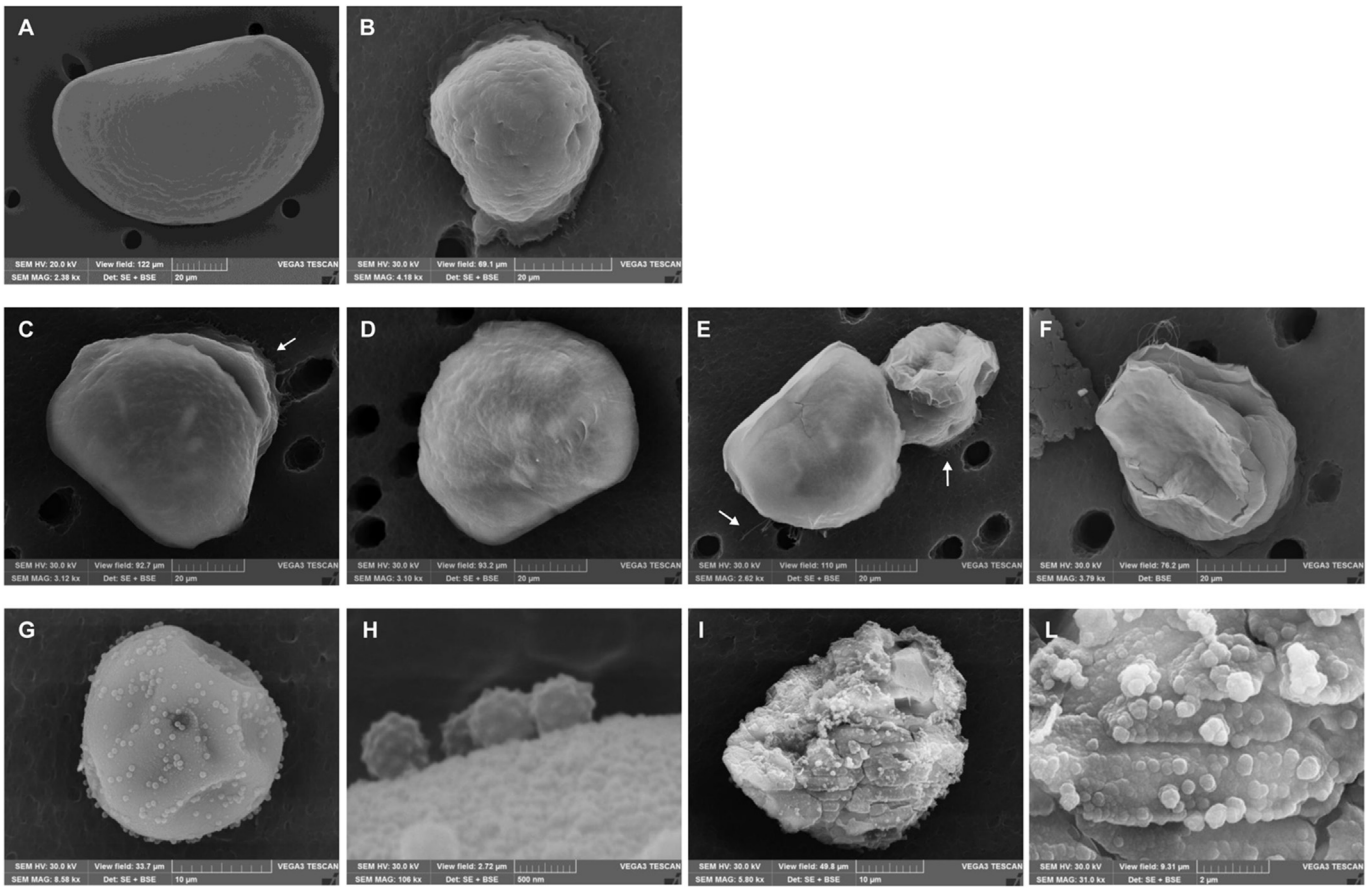

Fig. 4. Effects of exposure of fertilized eggs to higher concentrations of PS-NH_2 (5 mg/L) on the morphology of *Mytilus* embryos at 48 hpf evaluated by Scanning electron microscopy (SEM). A) control D-veliger at 48 hpf, with straight hinge, symmetric valvae and uniform surface. B-L) representative images of the different effects of PS-NH_2. B: embryo witheld at the trocophora stage; C,E; pre-veligers; D: malformed veliger. Note the present of thin, semi-transparent valvae with irregular shell surfaces. E, F: small malformed shells made of thin cracked lamellae. G,I: malformed embryo and D-shell of a dead embryo covered in small PS-NH_2 agglomerates with a rough (H) or a smooth surface (L). Arrows indicate the presence of externalized velum characteristic of pre-veligers.

both anionic and cationic PS NPs and n TiO_2 showed a comparable degree of agglomeration in marine water at concentrations between 0.1 and 1 mg/L (with the formation of stable agglomerates of about 200 nm in size) (Canesi et al., 2014; Della Torre et al., 2014), the results suggest that the positive surface charge retained by PS NH_2 in sea water, rather than core composition or differences in agglomeration state, may play a key role in determining the developmental effects of different types of NPs in marine in vertebrates. These data confirm that in marine organisms, like in mammalian systems, cationic NPs are generally more toxic with respect to anionic ones (Bexiga et al., 2011).

The cumulative effects of PS NH_2 on mussel embryo development were apparently independent on the timing of exposure. When in a separate set of experiments PS NH_2 was added either to fertilized eggs or to embryos at 24 hpf, at a concentration close to the EC_{50} (0.150 mg/L), no significant differences in the percentage of normal D veligers were observed at 48 hpf. However, exposure at 24 hpf induced a higher proportion of immature larvae, suggesting that the processes involved in the transition from the trocophora stage (24 hpf) to the first shelled embryo or D veliger (48 hpf) were mostly affected.

In order to gain an insight on the possible effects of PS NH_2 (0.150 mg/L) on shell formation, the degree of mineralization of mussel embryos at 48 hpf was estimated by polarized light microscopy. On the basis of the observed shell birefringence, which is due to the mineral phase, the larval area exhibiting birefringence can be interpreted to be covered by mineralized shell (Weiss et al., 2002). Bivalve larvae, including mussels, initially deposit amor phous calcium carbonate (ACC) in the shell (Weiss et al., 2002; Balbi et al., 2016), that is then partially transformed to aragonite, in contrast to adult shells that are predominantly composed of calcite. According to Weiss et al. (2002), bivalve larvae can be categorized into 3 types; fully mineralized (individuals that exhibit birefringence over the entire surface of the larva), partially mineralized (individuals in which only part of the larval surface exhibits birefringence), and non mineralized (larvae that exhibit no birefringence). Our data show a visible birefringence in control embryos, due to the presence of a partially mineralized shell. In samples exposed to PS NH_2, no birefringence was observed, indicating that exposure to PS NH_2 affects the mineralization process.

The possible mechanisms underlying the effects of PS NH_2were investigated by a transcriptomic approach. We have recently shown that in *M. galloprovincialis* significant increases in basal transcription of a number of genes involved in different biological functions occur across early developmental stages (eggs, 24 hpf, 48 hpf)(Balbi et al., 2016). In particular, the results underlined the role of genes involved in the initial formation of a first calcified shell, such as carbonic anhydrase (CA), that regulates matrix mineralization by generating an acidic environment (Clark et al., 2010) and *Mytilus* EP (Extrapallial Protein), an acidic calcium binding protein that regu lates the production of different polymorphs of calcium carbonate (Yin et al., 2009). In basal conditions, both EP and CA showed

highest expression at 48 hpf, confirming the key physiological role of both transcripts in initial biomineralization (Balbi et al., 2016). In this work, transcription of Chitin synthase (CS) was also evaluated. CSs are transmembrane glycosyltransferases that are responsible for the enzymatic synthesis of chitin, that not only represents a key structural component of the shell matrix, but also forms the framework for other macromolecules that guide initial shell deposition (Schonitzer and Weiss, 2007; Weiss and Schonitzer, 2006). Despite the importance of chitin synthesis in the formation of bivalve shells (Weiss et al., 2006), no information is available on expression of CS during early embryo stages. The results here obtained show that in control samples the level of transcripts for CS was slightly lower at 24 hpf with respect to the eggs, indicating that at this stage embryos still rely on maternal mRNA for this gene; however, a large increase in transcription of CS was recorded from 24 to 48 hpf, confirming the key role for chitin synthesis in the formation of the first shell.

In samples exposed to PS NH_2, at concentrations close to the EC_{50}, changes in transcription of CS, CA and EP were observed. Although a general transient up regulation was observed at 24 hpf, all genes were downregulated at 48 hpf. CS was the most affected gene, with significant changes at both times pf and, in particular, at 48 hpf, when the amount of mRNA was reduced by 50% with respect to controls, indicating impairment of the physiological production of chitin. In the same conditions, down regulation of CA and EP also indicate alterations in $CaCO_3$ deposition and mineralization.

Interestingly, PS NH_2 exposure also modulated expression of an *ABCB* transcript encoding the p glycoprotein transporter (P gp), with significant *ABCB* up regulation and down regulation at 24 and 48 hpf, respectively. The Multi xenobiotic resistance (MXR) system is considered a general and broad spectrum protective mechanism that consists of membrane and intracellular transporters acting as an active first tier defense against environmental chemicals, preventing their accumulation and toxic effects (Bielen et al., 2016). Among MXR related proteins in mussels, P gp is the best characterized. P gp is a membrane protein that mediates the direct extrusion of un metabolized xenobiotic out of the cell (i.e. phase 0 transporter) (Bard, 2000; Franzellitti and Fabbri, 2006). In adult mussels, induction of P gp has been reported in response to a wide range of chemical and physical stressors, suggesting that this transporter may be part of the general cellular stress response machinery. A recent study on expression and activity of MXR across *Mytilus* early development supported the hypothesis that in both embryos and adult mus sels P gp plays a prominent protective role against toxicants (Franzellitti et al., 2016). In particular, increased expression of *ABCB* and appearance of P gp mediated functional activity were detected at 48 hpf. The results of the present work suggest that exposure to PS NH_2 may impair the phase 0 of biotransformation of xenobiotics in D veligers. To our knowledge, this is the first report on the effects of NPs on expression of P gp transporters in a marine invertebrate embryo. In the sea urchin, much higher concentrations of PS NH_2 (3 mg/L) did not affect expression of *ABCB* transporters (Della Torre et al., 2014).

With regards to genes involved in the immune response, their transcription in bivalve development is generally low at early stages, and increases in later pre metamorphic stages, when larvae synthesize their own mRNA and proteins, acquiring immunocompetence (Balseiro et al., 2013; Song et al., 2016). Among these, Lysozymes are conserved enzymes that share the ability to split a (1,4) linkage between specific amino sugars, of the bacterial peptidoglycan. In suspension feeding bivalves, where bacteria also represent a source of food that is processed by intracellular digestion in the hepatopancreas, a molecular evolution of Lysozyme from a defense to a digestive function has been postulated, as observed in mammalian systems (Jollès et al., 1996). In adult *M. galloprovincialis* Lysozyme is expressed not only in immune cells, but also in the hepatopancreas and other tissues (Wang et al., 2012; Balbi et al., 2014). Our data show that in control embryos levels of mRNA for lysozyme were extremely low at 24 hpf in comparison to eggs; however, a large increase in tran scription was observed from 24 to 48 hpf. Exposure to PS NH_2 induced significant decreases in transcription of lysozyme at both times pf, indicating interference with the initial progression not only of immunocompetence, but also of processes related to the digestive function.

Interestingly, PS NH_2 did not affect transcription of a number of other genes involved in different key biological functions, such as the serotonin receptor 5 HTR and antioxidant enzymes, whose expression in early embryos was previously shown to be modulated by estrogenic compounds (Balbi et al., 2016). This lack of effects further underlines how, in *Mytilus* embryos, exposure to PS NH_2, at concentrations as low as 0.150 mg/L, specifically affected transcription of genes involved in shell formation, of *ABCB* transporters and lysozyme.

The effects of higher concentrations of PS NH_2 (5 mg/L) on larval morphology was also investigated by SEM as previously described (Balbi et al., 2016). The results confirm the presence of embryos blocked at the trocophora stage and malformed pre veligers, many of which had a shell made of thin cracked layers of mineralized lamellae, indicating that the process of shell formation was heavily affected in multiple ways depending on the concentration. Overall, the results confirm the biphasic effect PS NH_2 on embryo development at 48 hpf. Lower concentrations mainly induced shell malformations and affected crystallization of $CaCO_3$, whereas higher concentrations induced developmental arrest and high embryotoxicity.

The results of the present work represent the first indication on the molecular mechanisms involved in the possible developmental impact of NPs on bivalve molluscs. Overall, the obtained data underline that the transition from the trocophora stage (24 hpf) to the first shelled embryo D veliger (48 hpf) represents a critical stage for the effects of PS NH_2. Although a number of genes involved in biomineralization are being described in different bivalve species (Bassim et al., 2015; Vendrami et al., 2016), little information is available on those involved in the formation of a first calcified shell (prodissoconch I), a key step in early development. Our data demonstrate that the effects on PS NH_2 on D veligers were associated with down regulation of genes involved in the early processes of shell formation (i.e. chitin synthesis and $CaCO_3$ deposition). Interestingly, the effects of PS NH_2 were similar to those observed by exposure to high pCO_2 or low pH (Kadar et al., 2010; Kurihara et al., 2009), as well by pharmacological inhibition of chitin synthesis (Schonitzer and Weiss, 2007; Weiss and Schonitzer, 2006). Calcifying larvae of marine species are particularly vulnerable to abiotic stressors (Przeslawski et al., 2015). Our data further support the hypothesis that in *Mytilus* the transition from the trocophora stage to the first D shelled larva represents a most sensitive step to the action of contaminants (Balbi et al., 2016). In a global change scenario, concomitant changes in environmental parameters (i.e. acidification) and exposure to emerging contaminants such as NPs may represent a serious cause of concern for early life stages of sensitive marine species.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2017.07.120.

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