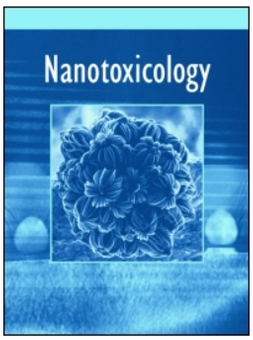


Nanotoxicology

ISSN: 1743-5390 (Print) 1743-5404 (Online) Journal homepage: http://www.tandfonline.com/loi/inan20

Amino-modified polystyrene nanoparticles affect signalling pathways of the sea urchin (Paracentrotus lividus) embryos

Annalisa Pinsino, Elisa Bergami, Camilla Della Torre, Maria Luisa Vannuccini, Piero Addis, Marco Secci, Kenneth A. Dawson, Valeria Matranga & Ilaria Corsi

To cite this article: Annalisa Pinsino, Elisa Bergami, Camilla Della Torre, Maria Luisa Vannuccini, Piero Addis, Marco Secci, Kenneth A. Dawson, Valeria Matranga & Ilaria Corsi (2017): Amino-modified polystyrene nanoparticles affect signalling pathways of the sea urchin (Paracentrotus lividus) embryos, Nanotoxicology, DOI: 10.1080/17435390.2017.1279360

To link to this article: http://dx.doi.org/10.1080/17435390.2017.1279360

Accepted author version posted online: 16 Jan 2017.
Published online: 24 Jan 2017.

NANOTOXICOLOGY, 2017
http://dx.doi.org/10.1080/17435390.2017.1279360

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Amino-modified polystyrene nanoparticles affect signalling pathways of the sea urchin (*Paracentrotus lividus*) embryos

Annalisa Pinsino[a], Elisa Bergami[b], Camilla Della Torre[c], Maria Luisa Vannuccini[b], Piero Addis[d], Marco Secci[d], Kenneth A. Dawson[e], Valeria Matranga[a] and Ilaria Corsi[b]

[a]CNR – Institute of Biomedicine and Molecular Immunology "A. Monroy", Palermo, Italy; [b]Department of Physical, Earth and Environmental Sciences, University of Siena, Siena, Italy; [c]Department of BioScience, University of Milan, Milan, Italy; [d]Department of Environmental and Life Sciences, University of Cagliari, Cagliari, Italy; [e]Centre for BioNano Interactions, School of Chemistry and Chemical Biology, University College Dublin, Dublin, Ireland

ABSTRACT

Polystyrene nanoparticles have been shown to pose serious risk to marine organisms including sea urchin embryos based on their surface properties and consequently behaviour in natural sea water. The aim of this study is to investigate the toxicity pathways of amino polystyrene nanoparticles (PS-NH_2, 50 nm) in *Paracentrotus lividus* embryos in terms of development and signalling at both protein and gene levels. Two sub-lethal concentrations of 3 and 4 μg/mL of PS-NH_2 were used to expose sea urchin embryos in natural sea water (PS-NH_2 as aggregates of 143 ± 5 nm). At 24 and 48 h post-fertilisation (hpf) embryonic development was monitored and variations in the levels of key proteins involved in stress response and development (Hsp70, Hsp60, MnSOD, Phospho-p38 Mapk) as well as the modulation of target genes (*Pl-Hsp70, Pl-Hsp60, Pl-Cytochrome b, Pl-p38 Mapk, Pl-Caspase 8, Pl-Univin)* were measured. At 48 hpf various striking teratogenic effects were observed such as the occurrence of cells/masses randomly distributed, severe skeletal defects and delayed development. At 24 hpf a significant up-regulation of *Pl-Hsp70, Pl-p38 Mapk*, *Pl-Univin and Pl-Cas8* genes was found, while at 48 hpf only for *Pl-Univin* was observed. Protein profile showed different patterns as a significant increase of Hsp70 and Hsp60 only after 48 hpf compared to controls. Conversely, P-p38 Mapk protein significantly increased at 24 hpf and decreased at 48 hpf. Our findings highlight that PS-NH_2 are able to disrupt sea urchin embryos development by modulating protein and gene profile providing new understandings into the signalling pathways involved.

ARTICLE HISTORY
Received 4 July 2016
Revised 10 November 2016
Accepted 4 December 2016

KEYWORDS
Sea urchin embryonic development; polystyrene nanoparticles; gene expression; signalling teratogenic effects

Introduction

Polystyrene (PS), an aromatic synthetic compound obtained by polymerisation of styrene monomers, represents one of the most widely used plastic, which has been released in the environment at an uncontrollable rate (Plastics – The Facts, 2015). PS is not readily degraded or processed by natural biological mechanisms (Andrady, 2011) but, instead, is broken up either mechanically or by photo- and thermal-oxidative degradation into plastic debris at the micrometric and nanometric size (Lambert & Wagner, 2016; Zhang et al., 2012).

Due to their small size, nanoplastics (<1 μm, as proposed by Browne et al., 2007; Hartmann et al., 2015) can be easily ingested and accumulated by organisms belonging to different trophic levels, with uncertain consequences for the health of the wildlife (Wright et al., 2013). Given the increasing evidence of plastic pollution in the ocean worldwide, their presence has become a significant environmental concern for governments, scientists and non-governmental organisations. For example, the EU Member States are committed to achieve or maintain good environmental status (GES) of their marine waters by 2020 through 11 qualitative descriptors, one of which (descriptor 10) is focused on marine litter, including plastics (Galgani et al., 2010; MSFD 2008/56/CE).

Several initiatives at scientific and industrial levels have improved the current knowledge in terms of risk assessment and sustainable productions. However, most of current information is on macro- and microparticles or fragments ingested by marine wildlife, possibly leading to damage to the digestive system and starvation or causing entanglement (Andrady, 2011; Kühn et al., 2015; Wright et al., 2013). Smaller fragments as nanoplastics are by far less studied, despite they can originate from the weathering and/or fragmentation of larger debris (macro and micro) mainly driven by movements of the oceans (horizontally and vertically) and by physico-chemical as well as biological parameters of the receiving sea waters. Due to their smaller size, nanoplastics can be taken up and accumulated by organisms. This makes their potential biological impact an important issue, given the current concerns regarding the environmental behaviour and ecotoxicity of nano-sized particles (Koelmans et al., 2015).

Nanoparticles (NPs) are well-known to affect marine organisms causing a wide variety of biological injuries in species belonging to different trophic levels, from planktonic to benthic species, including fish (Baker et al., 2014; Blasco et al., 2015; Canesi & Corsi, 2016; Matranga & Corsi, 2012; Minetto et al., 2014). Among them, nanoplastics recently attracted the attention of the scientific community since they have been shown to cause severe damages in marine invertebrates affecting immune system, survival and reproduction (Bergami et al., 2016; Canesi et al., 2015, 2016; Della Torre et al., 2014).

The enormous diversity of life in the sea offers a rich selection of organisms, especially invertebrates, with specialised adaptations

CONTACT Elisa Bergami elisa.bergami@student.unisi.it Department of Physical, Earth and Environmental Sciences, University of Siena, Siena, Italy

that enable researchers to learn more about the conserved molecular signalling pathways involved in protection, robustness, resistance and plasticity towards exposure to contaminants (Bodnar, 2016). Sea urchins have served as elective model in developmental biology research for more than a century, contributing to understand not only development and differentiation, but also the basis of cellular and molecular biology as well as evolutionary biology and most recently toxicology and ecotoxicology (Lawrence, 2013). Part of the sea urchin's value as a model organism is its close genetic relationship to humans, having an estimated 23,300 genes, with representatives of nearly all vertebrate gene families (Sea Urchin Genome Sequencing Consortium, 2006). Following the publication of the sea urchin genome, a new prospect seems to be opened in science: the use of sea urchin as an alternative *proxy to human* non-mammalian model to contribute to highlight the key protective molecules that could be used in innovative applications in environmental and biomedical fields. Considering its high sensitivity to low concentrations of contaminants and its discriminatory capability, the sea urchin is considered an excellent tool to assess the toxicity of many chemical compounds and environmental stressors, especially during its embryonic life stages (Morroni et al., 2016).

With this in mind, it provides an attractive and amenable model for exploring the molecular signalling pathways engaged in NPs recognition and interaction. Sea urchin embryo has been successfully used for assessing the toxicity of metal, metal-oxides and carbon-based NPs, showing to develop countless abnormalities depending on type, exposure conditions and concentrations (Burić et al., 2015; Canesi & Corsi, 2016; Corsi et al., 2014; Larson & Hutz, 2010; Pinsino et al., 2015; Šiller et al., 2013; Torres-Duarte et al., 2016; Wu et al., 2015).

For instance, Ag and SiO_2 NPs have been reported to cause dose-dependent developmental defects such as delayed development, bodily asymmetry, shortened or irregular arms and alterations in swimming patterns in embryos of the Mediterranean sea urchin *Paracentrotus lividus* (Gambardella et al., 2015; Siller et al., 2013). Conversely, in embryos of the white sea urchin *Lytechinus pictus,* both TiO_2 and CeO_2 NPs do no elicit visible harmful effects (Fairbairn et al., 2011).

We recently showed that PS NPs having cationic (–NH_2) or anionic (–COOH) surface groups undergo different aggregation in natural sea water (NSW), thus affecting their bio-distribution and ultimate toxicity in embryos of *P. lividus* (Della Torre et al., 2014).

Cationic PS-NH_2 have been shown to be cytotoxic for mammalian cells *in vitro*, while anionic PS-COOH exhibited greater and rapid accumulation in selected internal organs (Bexiga et al., 2011; Wang et al., 2013). Specifically, authors reported that PS-NH_2 toxicity depends on cell-specific endocytic and mitochondrial injury pathways involving caspases (i.e. caspase 3, 7, 9) (Bexiga et al., 2011; Xia et al., 2006). We hypothesised a similar mechanism of toxicity in sea urchin *P. lividus* embryos, since exposure to cationic PS-NH_2 induced developmental toxicity. Our first results on sea urchin embryos exposed to PS NPs suggested that PS-NH_2 (3 μg/mL nominal concentration) do not accumulate within cells/tissues during early development but cause teratogenesis, and stimulate an up-regulation of the *caspase 8* gene expression (24 h post fertilisation, hpf) (Della Torre et al., 2014). On the contrary, PS-COOH massively accumulates in the gut without producing any toxic effect to embryos. Studies in other marine invertebrate species confirm a significant accumulation of anionic PS NPs in the gut of rotifers (Snell & Hicks, 2011) and in bivalves (Ward & Kach, 2009; Wegner et al., 2012) as well as low toxicity.

Therefore, molecular mechanisms underlying the observed developmental disorder remain largely unknown and need to be investigated looking at signalling pathways elicited by PS-NH_2 during embryonic development. Since sea urchin embryos showed a discrete resistance and plasticity towards a persistent and direct interaction with PS-NH_2, we hypothesised that these particles are able to elicit a signalling regulating the balance between cell survival and death.

In order to test such hypothesis, in the present study, we investigated the effects of PS-NH_2 (50 nm) on sea urchin *P. lividus* embryonic development coupled with the variations in the levels of key proteins triggering stress response and development (Hsp70, Hsp60, MnSOD, P-p38 Mapk) as well as their gene expression profile (*Pl-Hsp70, Pl-Hsp60, Pl-Cytochrome b, Pl-p38 Mapk, Pl-Caspase 8, Pl-Univin*) under two PS-NH_2 sub-lethal concentrations of 3 and 4 μg/mL, previously shown to be able to affect embryonic development.

Materials and methods

NPs source and characterisation

Unlabelled PS-NH_2 (nominal size of 50 nm, positive surface charge) were purchased from Bangs Laboratories Inc (9025 Technology Dr, Fishers, IN 46038-2886 USA) and previously characterised both in Milli Q water (MilliQ, Merck KGaA, Darmstadt, Germany) and NSW by transmission electron microscopy (TEM) and dynamic light scattering (DLS), as described by Della Torre et al. (2014). TEM was applied for primary particle diameter identification of PS NPs (Philips Morgagni 268D electronics, at 80 KV and equipped with a MegaView II CCd camera). Dynamic Light Scattering (Malvern instruments) was used to determine size distribution parameters (*Z*-average and polydispersity index, PDI) (Zetasizer Nano Series software, version 7.02, Particular Sciences, UK). Measurements have been performed in triplicate, each containing 11 runs of 10 s.

PS-NH_2 suspensions were prepared in 0.45 μm filtered NSW (T 18 ± 1 °C, salinity 38‰, pH 8.3) and it was quickly vortexed prior to use. PS-NH_2 concentrations (3 and 4 μg/mL) used for embryotoxicity tests were chosen based on our previous findings on embryos of *P. lividus* (Della Torre et al., 2014), in which we observed several abnormalities at these low exposure concentrations (EC_{50} values of 3.85 and 2.61 μg/ml at 24 and 48 hpf, respectively) and also an apoptotic pathway at 3 μg/mL.

The NSW, obtained from a marine area located in South Sardinia (Italy), was previously 0.45 μm filtered and treated with Ultra Violet light before being used for experiments.

All exposure studies were carried out at the sea urchin hatchery facilities of the Department of Environmental and Life Science of the University of Cagliari (Italy). The following parameters of the NSW were maintained during experiments (0.45 μm filtration, 18 °C, 38‰, pH 8) and DLS analysis was performed in order to confirm that the aggregation state of PS-NH_2 was similar to that obtained in our previous study (Della Torre et al., 2014).

Sea urchin embryo culture and PS-NH_2 exposure

Paracentrotus lividus broodstock was induced to spawn by injection of 1 M KCl (40 μL per g of body weight) into the coelom via the peristomial membrane. The released eggs were fertilised by addition of 10 mL of diluted sperm (Carboni et al., 2013). Sea urchin embryos (600 embryos/mL) were exposed to 3 and 4 μg/mL PS-NH_2 suspensions soon after the fertilisation following the same procedure used in our previous experiments (Della Torre et al., 2014). Control embryos were exposed to NSW.

Table 1. List of primer sequences used for gene expression profile in sea urchin embryos investigated through real-time qPCR, including amplicon size (bp), efficiency (%), accession number and annealing temperature (°C).

Gene	Forward primer 5′–3′	Reverse primer 5′–3′	Amplicon size (bp)	Efficiency (%)	Acc number	Annealing T (°C)
Pl-Hsp70	CCAGTCGAAAGGGCAATCCT	CAACGTCGACGAGGAGAACA	244	100.5	X61379	59
Pl-Hsp60	GACGGAACCACAACAGCAAC	CCTGGGCGATTTCTTCTGGT	181	105.4	AJ249625	59
Pl-p38 Mapk	GTGATCAGCTTGCTTGACTG	GTAGATGAGGAACTGGACGTG	144	105.4	Marrone et al., 2012	58
Pl-14-3-3ε	GATCCCCTCTGCCAAAGACC	CCTGATAGGGTCTGTCGGGT	187	101.8	AJ493680	61
Pl-Univin	GATCATCGCTCCGATGGGTT	GCTCGGCTATCGATGGAGTT	129	94.5	AJ302364	59
Pl-Caspase 8	TCCTGCCCAAACTGCTACTG	CCTCGCAGTTGGGTCTTTCT	232	99.0	EU078681	59
Pl-Cytb	GGGATACGTATTAGTCTGAGG	CGAGTTAGGGTGGCATTGTC	144	108.8	Marrone et al., 2012	58
Pl-18S	GAATGTCTGCCCTATCAACTTTCG	TTGGATGTGGTAGCCGTTTCTC	119	104.0	Ragusa et al., 2013	60

At key time-points of the development as 24 hpf (gastrula stage) and 48 hpf (pluteus stage), embryo cultures (40 mL; about 24000 embryos) were collected by centrifugation (2000 *g* for 8 min), stored in liquid nitrogen and further at −80 °C for protein and gene expression analyses. Embryos were monitored to assess the occurrence and timing of several morphological events: endoderm, ectoderm and mesoderm (germ layers) development and differentiation. Aliquots of 5 mL of embryos at both 24 hpf and 48 hpf were fixed in 1:1 lugol:ethanol 70%, inspected by optical microscopy (Olympus BX51) and photographed by a DP50 digital camera.

SDS-PAGE, immunoblotting

Lyophilised pellets were Dounce-homogenized on ice in about 250 μL of lysis buffer [20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton], supplemented with a protease inhibitor cocktail (Roche) and phoshatase inhibitor cocktail (SIGMA). Total cell lysate (30 μg) per each cell extract was run on a 4–15% Mini-PROTEAN TGX precast polyacrylamide gels (Bio-Rad) and transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Amersham UK). After blocking, replicate membranes were incubated overnight at 4 °C with either one of the following primary antibodies: (i) Heat Shock Protein 70 (SIGMA, Cat N. H-5147) 1:1000; (ii) Heat Shock Protein 60 (SIGMA, Cat N. H-3524) 1:250; (iii) Phospho-p38 MAP Kinase (Thr180/Tyr182) (Cell Signalling, 9211) 1:250; (iv) Manganese superoxide dismutase (Enzo Life Sciences, ADI-SOD-110) 1:500. Membranes were washed three times with PBS-Tween 20 prior to incubation with a fluorescein-labelled secondary anti-mouse and/or -rabbit antibody (LI-COR Biosciences). Protein levels (Hsp70, Hsp60, MnSOD, P-p38 Mapk) were normalised using α-tubulin as internal control. Results were reported in arbitrary units ± SE obtained from the volumetric analysis of the normalised bands (sum of the two independent experiments).

RNA extraction, cDNA synthesis, real-time quantitative PCR

Total RNA was extracted from pellets of control and exposed embryos (3 and 4 μg/mL PS-NH_2) from specimens obtained from two independent experiments. Total RNAs from each specimen (250 ng) were reverse transcribed using I-script cDNA Reverse Transcription Kit (Biorad), according to manufacturer's instructions. The real-time qPCRs were run using a StratageneMx3000P thermal cycler, as previously described (Della Torre et al., 2014). The expression of selected genes, which are known to be involved in the development and stress-response (*Pl-Hsp70, Pl-Hsp60, Pl-Cytb, Pl-p38 Mapk, Pl-Caspase 8, Pl-Univin*) was investigated. The *Pl-18S* was used as internal endogenous reference gene. *Pl-p38 Mapk, Pl-Cytochrome b* and *Pl-18S* primer sequences were already published (Marrone et al., 2012; Ragusa et al., 2013), whereas others were designed using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences used for real-time qPCRs and the amplicon lengths (ranging from 129 to 244 bp) are summarised in Table 1. Data were analysed by the ΔΔCt method (Pfaffl 2001). The levels of expression of the genes were considered significantly different when they were two-fold higher or lower than those measured in controls (0.5 < not significant <2).

Statistical analysis

All data were analysed by the one-way analysis of variance (one-way ANOVA) compared with the respective control group, followed by the multiple comparison test of Tukey's, using the OriginPro 7.5 statistical programme (the level of significance was set as $p \leq 0.05$).

Results and discussion

PS-NH_2 characterisation

PS-NH_2 nominal diameter of 50 nm in MilliQ was confirmed by TEM. Secondary characterisation of PS-NH_2 by DLS revealed the formation of a number of small aggregates in NSW (*Z*-average of 143 ± 5 nm SD and PDI of 0.413), slightly higher with respect to the average aggregate size reported in our previous study for the same particle (89 ± 2 nm, Della Torre et al., 2014). The limited increase in the average size of PS-NH_2 aggregates could be related to the different NSW used (see Materials and Methods section). However, no differences in phenotypes of sea urchin embryos at 24 and 48 hpf previously exposed (Della Torre et al., 2014) and those obtained in the present study were observed.

PS-NH_2 effects on sea urchin embryonic development

At 24 hpf, at both concentrations used, embryos displayed abnormal proliferation of ectodermal membrane despite they maintained regular time schedule and proper sites of spicule elongation (Not shown). Such phenotypes were in agreement with our previous findings in which the thickening and abnormal proliferation was observed at this developmental stage (Della Torre et al., 2014).

At the pluteus stage (48 hpf), PS-NH_2 exposed embryos displayed a high number of abnormalities, defined as delayed in the developmental schedule or impairment of the correct differentiation of the derivatives germ layers (Figure 1).

Specifically, more than 70% of embryos exposed to 3 μg/mL PS-NH_2 belonged to the delayed category, showing a correct morphology with a pyramidal shape although they were late in skeleton elongation (see black and pink arrow heads in Figure 1(B–B′)) compared to controls (Figure 1(A–A′)).

An increased incidence of potential teratogenic effects was observed at 4 μg/mL PS-NH_2. At this concentration, 90% of embryos exhibited poorly developed skeleton rods,

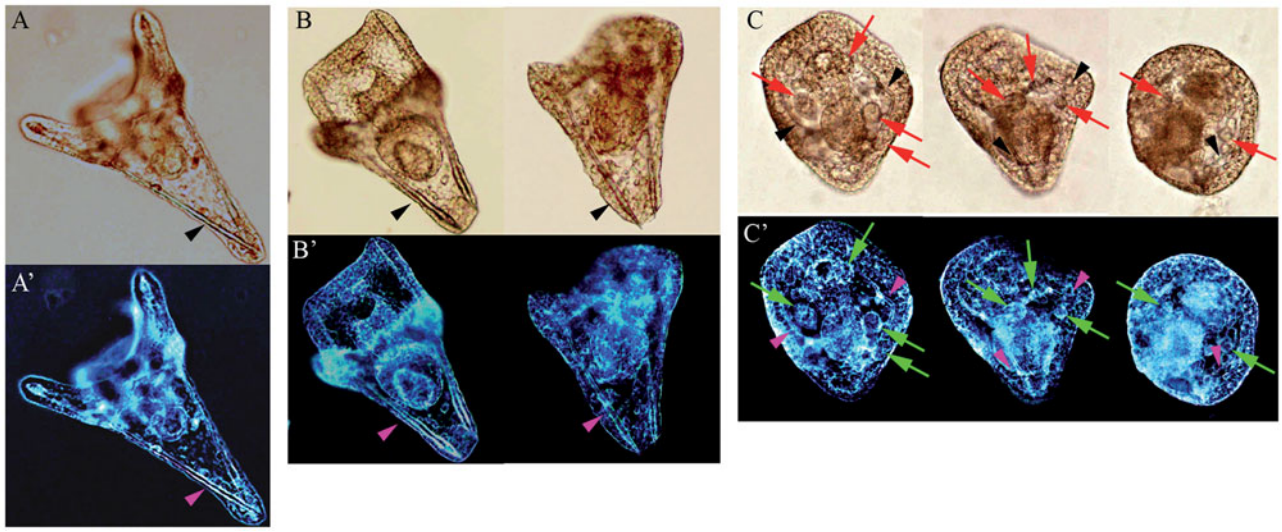


Figure 1. PS-NH_2-exposed embryos under optical microscopy. Pictures of representative sea urchin *P. lividus* embryos exposed to PS-NH_2 (3 and 4 μg/mL) are shown. Control (A–A′) and 3 and 4 μg/mL PS-NH_2-exposed (B–B′ and C-C′) embryos at pluteus stage (48 hpf). PS-NH_2 exposure produced teratogenic effects in the sea urchin embryos. Embryos exposed to 3 μg/mL PS-NH_2 belonged to the delayed category, showing a correct morphology with a pyramidal shape although they were late in skeleton elongation (black and pink arrows). Embryos exposed to 4 μg/mL PS-NH_2 revealed poorly developed skeleton rods, underdeveloped/poorly developed arms, poor symmetry and atypical cells/masses randomly distributed, which appeared bigger than expected (red and green arrows). Arrows indicate skeleton (black and pink arrows) and atypical cells/masses (red and green arrows). (For the interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

underdeveloped/poorly developed arms, poor symmetry and a typical cells/masses randomly distributed, which appeared bigger than expected (see red and green arrows Figure 1(C–C′)).

Similar skeleton-defective embryos have been previously reported for *P. lividus* exposed to other types of NPs suggesting that such abnormalities in embryo phenotype could be used as sensitive target upon NP exposure (Gambardella et al., 2013, 2015). A competition with calcium uptake, internalisation and consequently with bio-mineral formation during development could be responsible for such abnormalities as previously postulated in manganese-exposed embryos (Pinsino et al., 2011a).

Besides, the formation of typical cells/masses as shown upon PS-NH_2 exposure is in agreement with results previously obtained by Magesky and Pelletier (2015) in *Strongylocentrotus droebachiensis* embryos exposed to Ag NPs.

Due to their random distribution, it can be hypothesised that, in 4 μg/mL PS-NH_2 exposed embryos, these big cells/masses could be putatively intoxicated blastocoelar cells or other non-skeletogenic mesodermal cells appearing malformed as though they were vacuolated or swelled. Normal blastocoelar cells are embryonic phagocytes with a fusiform aspect residing in the blastocoel, particularly concentrated around the gut (Tamboline & Burke, 1992). As phagocytosis is an early line of defence against host invasion, and is known mechanism of NP internalisation (Petros & De Simone, 2010), blastocoelar cells could represent a primary cellular target of PS-NH_2. However, our results do not rule out the possibility that other embryonic cell types are also able to internalise PS-NH_2 involving phagocytosis or other cellular pathways.

On the other hand, PS-NH_2 could interfere indirectly with the normal embryonic development altering the biological culture media in which the embryo grows. For example, NP agglomerates/aggregates surrounding embryonic tissues could be able to interact with the cell membranes, disrupting the membrane functions (i.e. ion transport or signal transduction) (Fleischer & Payne, 2012; Rossi et al.. 2014; Salvati et al., 2011) and inducing teratogenic effects (Bexiga et al., 2011; Canesi et al., 2015; Della Torre et al., 2014; Wang et al., 2013). Further studies are needed to clarify at least one of these hypotheses.

During embryonic development, cells proliferate rapidly and differentiate to form specialised cells, most of which enter and stay into the form of G_0 phase. These cells are able to restart proliferation when required to replace cells after injury or cell death, activating alternative signalling pathways as a defence strategy for embryonic survival (Hamdoun & Epel, 2007).

In the sea urchin embryo, an integrated network of genes, proteins and pathways defends itself against chemical agents (Defensome). This network either buffers stress or produces alternative developmental phenotypes (Hamdoun & Epel, 2007).

Since PS-NH_2 exposure up to 4 μg/mL significantly interfered with the sea urchin embryonic development, producing teratogenic but not lethal effects, it can be postulated that PS-NH_2 are able to elicit signalling pathways regulating the balance between embryonic cell survival and death.

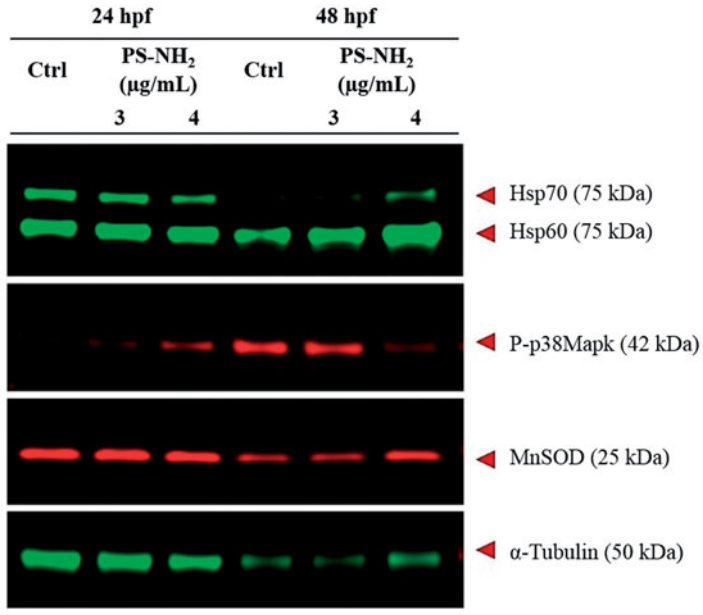
Molecular machinery engaged in PS-NH_2 exposure

To get a deeper insight into the molecular mechanisms underlying the effects of PS-NH_2 on sea urchin embryonic development, variations in the levels of key proteins triggering stress response and development (Hsp70, Hsp60, MnSOD, P-p38 Mapk) as well as in the expression of target genes (*Pl-Hsp70, Pl-Hsp60, Pl-Cytb, Pl-p38 Mapk, Pl-Caspase 8, Pl-Univin*) were analysed.

Proteins and genes were chosen taking into consideration: (i) the protein functions; (ii) the cellular localizations; (iii) the processes in which proteins participate during embryonic development; (iv) the biological and molecular results obtained from studies performed in the sea urchin embryos exposed to several chemical and physical stressors, including NPs.

For example, the stress proteins belonging to the Hsp70 and Hsp60 family are a collective of specialised proteins, molecular chaperones, which bind non-native states of other proteins and assist them to reach a functional conformation (Bukau & Horwich, 1998). MnSOD is a nuclear-encoded and mitochondria-matrix-

(A)



(B)

Fold variation vs Ctrl	24 hpf		48 hpf	
	PS-NH$_2$ (μg/mL)		PS-NH$_2$ (μg/mL)	
	3	4	3	4
Hsp70	1±0.06	0.7±0.03	**1.5±0.05***	**3±0.5***
Hsp60	1.41±0.2	1.2±0.03	**2.13±0.13***	**3.75±0.25***
MnSOD	1.14±0.04	1.05±0.16	1.24±0.04	1.2±0.05
p-p38 Mapk	**2±0.17***	**2.68±0.16***	1.3±0.29	**4±0.05***

Figure 2. Levels of key proteins triggering stress response and development after PS-NH$_2$ exposure. (A) Representative image of the Hsp70, Hsp60, P-p38 MAPK and MnSOD protein levels of control and PS-NH$_2$ exposed embryos (3 and 4 μg/mL) at 24 and 48 hpf evaluated by immunoblotting. (B) Table showing fold variation (as mean ± SE) of the proteins by increasing PS-NH$_2$ concentration from 3 to 4 μg/mL, compared to control. Data are representative of two independent experiments after normalisation with α-tubulin levels (assumed constant). *Significant difference with respect to controls.

localized oxidation-reduction (redox) enzyme that regulates cellular redox homeostasis (Sarsour et al., 2014), while the Cytb is one of the major enzymes responsible for mitochondrial superoxide production (Zhu et al., 1999). A number of published studies demonstrated that, in the sea urchin embryos, these molecules are involved in the activity of intracellular signalling molecules, determining stress resistance, immune resistance and apoptosis (Bonaventura et al., 2011; Korkina et al., 2000; Marrone et al., 2012; Pinsino et al., 2010, 2011b; Roccheri et al., 2004). However, to our knowledge, only our previous study aimed to analyse gene expression profile upon PS NPs exposure in sea urchin embryo (Della Torre et al., 2014). Here, we extended this aim by focusing on key components of the signalling regulating growth and survival in sea urchin embryos at 24 and 48 hpf using sub-lethal concentrations of the PS-NH$_2$ (3 and 4 μg/mL).

Specifically, after 24 hpf, PS-NH$_2$ exposure (3 μg/mL concentration) was not effective in inhibiting or enhancing the protein levels of Hsp70, Hsp60 and MnSOD, respectively (see Figure 2(B)). At the same time, PS-NH$_2$ exposed embryos displayed a two-fold increase in the P-p38 Mapk protein levels with respect to controls (p_{value} = .00437). PS-NH$_2$ exposed embryos showed levels of expression of the *Pl-Hsp70* gene 3.9-fold higher than those measured in the controls (assumed as 1), while the transcripts of *Pl-Hsp60* and *Pl-Cytb* did not show a significant difference (Figure 3(A)). In addition, an increase in the levels of expression of the *Pl-Univin* and *Pl-Cas8* genes (2- and 2.8-fold) was also found (Figure 3(A)).

At 24 hpf, embryos exposed to 4 μg/mL nominal concentration showed significantly higher levels of the *Pl-Hsp70, Pl-p38 Mapk*, *Pl-Univin* and *Pl-Cas8* gene expression compared to controls (3.3, 3.5, 2.2, 2.8 and 2.3 fold, respectively), while the transcripts of *Pl-Hsp60* and *Pl-Cytb* did not show any change (Figure 2(B)). In accordance, no significant differences in the levels of the Hsp70, Hsp60 and MnSOD proteins were observed in PS-NH$_2$-exposed embryos. Instead, embryos displayed an increase in the P-p38 Mapk protein levels higher than those measured in the 3 μg/mL PS-NH$_2$-exposed embryos (2.7-fold) (p_{value} = 0.00047) (Figure 2(B)).

In summary, at 24 hpf embryos exposed to 3 and 4 μg/mL show: (i) similar levels of expression of the *Pl-Hsp70* gene (from 3.93- to 3.33-fold); (ii) levels of expression of *Pl-p38* Mapk and *Pl-Univin* genes increased with increasing concentration (from 1.53- to 3.58-fold and from 1.98 to 2.22); (iii) levels of expression of *Pl-Cas8* unchanged; (iv) any modulation of Hsp70/Hsp60/MnSOD proteins and (v) a dose-dependent increase of p38 Mapk protein phosphorylation.

Our findings indicate that at 24 hpf, PS-NH$_2$ were not effective in modulating proteins having stress-related function (Hsp70/

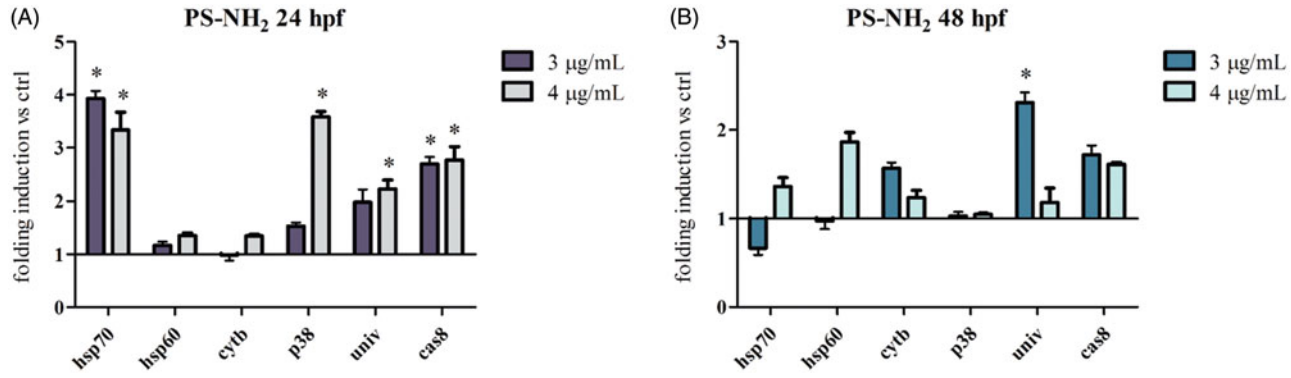


Figure 3. Misregulation of gene expression in response to PS-NH_2 exposure. Levels of expression of *Pl-Hsp70, Pl-Hsp60, Pl-Cytochrome b, Pl-p38 Mapk, Pl-Caspase 8, Pl-Univin* genes analysed by comparative real-time qPCR with total RNA isolated from control and exposed embryos at (A) 24 hpf and (B) 48 hpf. Results are expressed as fold increase compared to controls assumed as 1, using the endogenous gene The *Pl-18S* for normalisation. Each bar represents the mean of two independent experiments ± SE. *Significant difference respect to controls.

Hsp60/MnSOD) in agreement with our previous observations with other types of external stimuli (i.e. metals, radiations) (Bonaventura et al., 2011; Pinsino et al., 2010). On the contrary, a significant increase of P-p38 Mapk protein was observed. The p38 Mapk is a member of a well-studied family of proteins that plays an essential role in the signalling events associated to survival response, apoptosis and autophagy, but it is also known as key regulator of both embryonic development and cancer progression (Bradham & McClay, 2006; Pinsino et al., 2014).

These results were consistent with report describing manganese effects on p38 Mapk phosphorylation during sea urchin embryonic development, in which we emphasised the role played by p38 Mapk in embryo skeleton development (Pinsino et al., 2014).

The up-regulation of Univin observed in sea urchin embryos upon PS-NH_2 exposure highlighted skeletal development as potential target of toxicity, supported also by the evident skeletal defects observed in embryos exposed to 4 µg/mL. Univin is a member of the transforming growth factor beta (TGF-beta) protein superfamily, known to be one of the promoter of the skeletal growth in the sea urchin embryos (Zito et al., 2003). Specifically, Univin is a relevant Alk4/5/7 ligand for anterior skeletal patterning during gastrulation (Piacentino et al., 2015).

Furthermore, Caspase 8 is known to be implicated in the apoptosis regulation but also required for the development of specific embryonic cell types (Fu et al., 2000; Kruidering & Evan, 2000). Taken together, our results suggest that at 24 hpf, PS-NH_2 activate protein-coding genes, involved both in embryos survival and development.

Concerning effects observed at 48 hpf, embryos exposed to 3 and 4 µg/mL PS-NH_2 displayed a concentration-dependent increase in the levels of the Hsp60 protein compared to controls (2-fold and 3.8-fold, respectively) ($p_{value} = 0.00283$). A slight increase in *Pl-Hsp60* gene expression (1.9-fold) only at the highest concentration was observed (Figure 2(A) and 3(B)). This result suggests that the stress-induced Hsp60 could be regulated either at the transcriptional level by activation of the heat shock transcription factors (HSFs) or at the translational level, irrespectively of the HSF transcriptional activity. Gene expression studies in cancer revealed an interruption between transcription and translation of Hsp60. For instance, the increased expression of the *Hsp60* gene is observed in different types of human cancers (ovarian cancer, pancreatic cancer, large bowel carcinoma, etc.) due to the direct regulation of *Hsp60* expression by *c-Myc* (Tsai et al., 2008).

The increase of the *Hsp60* gene, responsible for the cell transformation could be linked to the presence of atypical cells/masses randomly distributed in embryos exposed to 4 µg/mL PS-NH_2.

On the other hand, the increase in the levels of the Hsp60 protein suggests the activation of a cell survival programme focused on stabilisation of mitochondrial proteins. This finding is not surprising, given that at this stage of development we also found a slight increase in the expression levels of the *Pl-Caspase 8* gene (1.8 and 1.7 fold) (Figure 3(B)). Caspase 8 would serve to counteract tumorigenesis and ensure survival. Furthermore, a concentration-dependent increase in the levels of the Hsp70 protein was observed in embryos exposed to 3 and 4 µg/mL PS-NH_2 when compared to controls (1.5- and 3-fold, respectively) ($p_{value} = 0.03327$). On the contrary, no appreciable variation in the expression levels of the *Pl-Hsp70* gene was found (Figure 3(B)).

These findings are consistent with the notion that, under conditions of prolonged stress, HSF (the transcription factor that regulates the expression of all heat shock genes) is inactivated by negative feedback mechanisms (Voellmy, 2004). Specifically, increased levels of the Hsp70 protein cause the inactivation of own transcription factor.

In addition, in line with the results obtained at 24 hpf, PS-NH_2 exposure was not effective in inhibiting or enhancing the protein levels of MnSOD and the transcripts of *Pl-Cytb*, suggesting that PS-NH_2 exposure do not provoke oxidative stress during embryonic development.

Differently, the P-p38 Mapk protein levels were found significantly decreased at the highest concentration used (4-fold) compared to controls (Figure 2(A,B)). A recent study showed that the p38 Mapk inhibition markedly reduces the tumorigenicity by modulating angiogenesis, cell motility and invasion (Marengo et al., 2013). As reported for *Pl-Hsp70* gene, also in this case the levels of expression of the *Pl-p38 Mapk* gene were found comparable to controls. Finally, exposed embryos showed levels of expression of the *Pl-Univin* gene significantly different from those measured in controls only at the lowest concentration (Figure 3(B)).

In summary, overall results highlighted an increase in embryotoxicity towards PS-NH_2 exposure at pluteus stage (48 hpf) at relatively low concentrations with the involvement of molecular targets such as P-p38 Mapk, Hsp60 and Hsp70 proteins, *Pl-Hsp60* and *Pl-Univin* genes.

Conclusions

Overall, results highlighted an astounding level of developmental plasticity of the sea urchin embryos exposed to PS-NH_2, established by the ability to try to maintain the underlying developmental pathways and to activate alternative signalling

pathways as a defence strategy for survival to these nanoplastics.

Sea urchin embryos plasticity to environmental changes and emerging contaminants should not be undervalued. The importance of our study on sea urchin embryos is therefore 2-fold as it provides a powerful model for nano-toxicity/nano-safety investigations as a proxy *to humans* confirming themselves as elective model for ecotoxicological studies.

Our study confirmed the concern related to the presence of nanoplastics in the marine environment. PS-based NPs are able to elicit several developmental defects, despite the potent cellular defence machinery against environmental challenges present in embryos. Our findings stress the need to thoroughly characterising the occurrence and environmental fate of nanoplastics in marine ecosystems, and to assess the interactions with marine wildlife, focusing on misregulation of key signalling pathways involved in the onset of toxicity. The information thus obtained can be useful for defining predictive investigative tools for the evaluation of the biological risk related to these emerging environmental pollutants.

Acknowledgements

This paper is dedicated to Valeria Matranga, prematurely passed away in April this year, after a long and courageous battle against illness. In the last thirty years, Valeria's work contributed immensely to our understanding of fundamental cellular and molecular processes in the sea urchin model. We thank her for devotion, enthusiasm in research and great collaborative and welcoming approach.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

References

Andrady AL. 2011. Microplastics in the marine environment. Mar Pollut Bull 62:1596–605.

Baker TJ, Tyler CR, Galloway TS. 2014. Impacts of metal and metal oxide nanoparticles on marine organisms. Environ Pollut 186:257–71.

Bergami E, Bocci E, Vannuccini ML, Monopoli MP, Salvati A, Dawson KA, Corsi I. 2016. Nano-sized polystyrene affects feeding, behavior and physiology of brine shrimps Artemia franciscana larvae. Ecotoxicol Env Saf 123:18–25.

Bexiga MG, Varela JA, Wang F, Fenaroli F, Salvati A, Lynch I, et al. 2011. Cationic nanoparticles induce caspase 3-, 7- and 9-mediated cytotoxicity in a human astrocytoma cell line. Nanotoxicology 5:557–67.

Blasco J, Corsi I, Matranga V. 2015. Particles in the oceans: implication for a safe marine environment. Mar Environ Res 111:1–4.

Bodnar A. 2016. Lessons from the sea: marine animals provide models for biomedical research. Environ SciPolicy Sustain Dev 58:16–25.

Bonaventura R, Zito F, Costa C, Giarrusso S, Celi F, Matranga V. 2011. Stress response gene activation protects sea urchin embryos exposed to X-rays. Cell Stress Chaperones 16:681–7.

Bradham C, McClay DR. 2006. p38 MAPK in development and cancer. Cell Cycle 5:824–8.

Browne MA, Galloway T, Thompson R. 2007. Microplastic-an emerging contaminant of potential concern? Integr Environ Assess Manag 3:559–61.

Bukau B, Horwich AL. 1998. The Hsp70 and Hsp60 chaperone machines. Cell 92:351–66.

Burić P, Jakšić Ž, Štajner L, Dutour Sikirić M, Jurašin D, Cascio C, et al. 2015. Effect of silver nanoparticles on Mediterranean sea urchin embryonal development is species specific and depends on moment of first exposure. Mar Environ Res 111:50–9.

Canesi L, Ciacci C, Bergami E, Monopoli MP, Dawson KA, Papa S, et al. 2015. Evidence for immunomodulation and apoptotic processes induced by cationic polystyrene nanoparticles in the hemocytes of the marine bivalve Mytilus. Mar Environ Res 111:34–40.

Canesi L, Corsi I. 2016. Effects of nanomaterials on marine invertebrates. Sci Total Environ S0048-9697:30086–9.

Canesi L, Ciacci C, Fabbri R, Balbi T, Salis A, Damonte G, et al. 2016. Interactions of cationic polystyrene nanoparticles with marine bivalve hemocytes in a physiological environment: role of soluble hemolymph proteins. Environ Res 150:73–81.

Carboni S, Hughes AD, Atack T, Tocher DR, Migaud H. 2013. Influence of broodstock diet on somatic growth, fecundity, gonad carotenoids and larval survival of sea urchin. Aquac Res 46:1–8.

Corsi I, Cherr GN, Lenihan HS, Labille J, Hassellov M, Canesi L, et al. 2014. Common strategies and technologies for the ecosafety assessment and design of nanomaterials entering the marine environment. ACS Nano 8:9694–709.

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

Fairbairn EA, Keller AA, Mädler L, Zhou D, Pokhrel S, Cherr GN. 2011. Metal oxide nanomaterials in seawater: linking physicochemical characteristics with biological response in sea urchin development. J Hazard Mater 192:1565–71.

Fleischer CC, Payne CK. 2012. Nanoparticle surface charge mediates the cellular receptors used by protein-nanoparticle complexes. J Phys Chem B 116:8901–7.

Fu H, Subramanian RR, Masters SC. 2000. 14-3-3 proteins: structure, function, and regulation. Annu Rev Pharmacol Toxicol 40:617–47.

Galgani F, Fleet D, Van Franeker J, Katsanevakis S, Maes T, Mouat J, et al. 2010. Marine Strategy Framework Directive – Task Group 10 Report Marine litter. JRC Scientific and Technical Reports 1 – 57. JRC 58104 EUR 24340 EN. DOI 10.2788/86941.

Gambardella C, Aluigi MG, Ferrando S, Gallus L, Ramoino P, Gatti AM, et al. 2013. Developmental abnormalities and changes in cholinesterase activity in sea urchin embryos and larvae from sperm exposed to engineered nanoparticles. Aquat Toxicol 130–131:77–85.

Gambardella C, Morgana S, Bari GD, Ramoino P, Bramini M, Diaspro A, et al. 2015. Multidisciplinary screening of toxicity induced by silica nanoparticles during sea urchin development. Chemosphere 139:486–95.

Hamdoun A, Epel D. 2007. Embryo stability and vulnerability in an always changing world. Proc Natl Acad Sci USA104:1745–50.

Hartmann N, Nolte T, Sørensen M, Jensen P, Baun A. 2015. Aquatic ecotoxicity testing of nanoplastics. Lessons Learned From Nanoecotoxicology. DTU Environment.

[Online] Available at: http://www.ncbi.nlm.nih.gov/tools/primer-blast/

Koelmans AA, Besseling E, Shim WJ. 2015. Nanoplastics in the aquatic environment. Critical review. In: Bergmann M, Gutow L, Klages M, eds. Marine Anthropogenic Litter. Cham: Springer, 325–440

Korkina LG, Deeva IB, De Biase A, Iaccarino M, Oral R, Warnau M, Pagano G. 2000. Redox-dependent toxicity of diepoxybutane and mitomycin C in sea urchin embryogenesis. Carcinogenesis 21:213–20.

Kruidering M, Evan GI. 2000. Caspase-8 in apoptosis: the beginning of IUBMB Life 50:85–90.

Kühn S, Bravo Rebolledo EL, van Franeker JA. 2015. Deleterious effects of litter on marine life. In: Bergmann M, Gutow L, Klages M, eds. Marine Anthropogenic Litter. Cham: Springer, 75–116.

Lambert S, Wagner M. 2016. Characterisation of nanoplastics during the degradation of polystyrene. Chemosphere 145:265–8.

Larson JK, Hutz RJ. 2010. Gold nanoparticles alter sea urchin development to pluteus stage. Biol Reprod 83:285.

Lawrence JM. 2013. Sea urchins: Biology and Ecology. 3rd ed. London: Elsevier, 550.

Magesky A, Pelletier É. 2015. Toxicity mechanisms of ionic silver and polymer-coated silver nanoparticles with interactions of functionalized carbon nanotubes on early development stages of sea urchin. Aquat Toxicol 167:106–23.

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

Marengo B, De Ciucis CG, Ricciarelli R, Furfaro AL, Colla R, Canepa E, et al. 2013. p38MAPK inhibition: a new combined approach to reduce neuroblastoma resistance under etoposide treatment. Cell Death Dis 4:e589.

Marrone V, Piscopo M, Romano G, Ianora A, Palumbo A, Costantini M. 2012. Defensome against toxic diatom aldehydes in the sea urchin *Paracentrotus lividus*. PLoS One 7:e31750.

Minetto D, Libralato G, Volpi Ghirardini A. 2014. Ecotoxicity of engineered TiO2 nanoparticles to saltwater organisms: An overview. Environ Int 66:18–27.

Morroni L, Pinsino A, Pellegrini D, Regoli F, Matranga V. 2016. Development of a new integrative toxicity index based on an improvement of the sea urchin embryo toxicity test. Ecotoxicol Environ Saf 123:2–7.

MSFD, Marine Strategy Framework Directive, 2008/56/CE Official Journal of the European Union, L. 164/19.

Petros RA, De Simone JM. 2010. Strategies in the design of nanoparticles for therapeutic applications. Nat Rev Drug Discov 9:615–27.

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucl Ac Res 29:2002–7.

Piacentino ML, Ramachandran J, Bradham CA. 2015. Late Alk4/5/7 signaling is required for anterior skeletal patterning in sea urchin embryos. Development 142:943–52.

Pinsino A, Matranga V, Trinchella F, Roccheri MC. 2010. Sea urchin embryos as an in vivo model for the assessment of manganese toxicity: developmental and stress response effects. Ecotoxicology 19:555–62.

Pinsino A, Roccheri MC, Costa C, Matranga V. 2011a. Manganese interferes with calcium, perturbs ERK signaling, and produces embryos with no skeleton. Toxicol Sci 123:217–30.

Pinsino A, Turturici G, Sconzo G, Geraci F. 2011b. Rapid changes in heat-shock cognate 70 levels, heat-shock cognate phosphorylation state, heat-shock transcription factor, and metal transcription factor activity levels in response to heavy metal exposure during sea urchin embryonic development. Ecotoxicology 20:246–54.

Pinsino A, Roccheri MC, Matranga V. 2014. Manganese overload affects p38 MAPK phosphorylation and metalloproteinase activity during sea urchin embryonic development. Mar Environ Res 93:64–9.

Pinsino A, Russo R, Bonaventura R, Brunelli A, Marcomini A, Matranga V. 2015. Titanium dioxide nanoparticles stimulate sea urchin immune cell phagocytic activity involving TLR/p38 MAPK-mediated signalling pathway. Sci Rep 5:14492

Plastics – The Facts. 2015. An analysis of European plastics production, demand and waste data, Brussels, Belgium: Plastics Europe. [Online] Available at: 2015. http://www.plasticseurope.org/Document/plastics—the-facts-2015.aspx

Ragusa MA, Costa S, Gianguzza M, Roccheri MC, Gianguzza F. 2013. Effects of cadmium exposure on sea urchin development assessed by SSH and RT-qPCR: metallothionein genes and their differential induction. Mol Biol Rep 40:2157–67.

Roccheri MC, Agnello M, Bonaventura R, Matranga V. 2004. Cadmium induces the expression of specific stress proteins in sea urchin embryos. Biochem Biophys Res Commun 321:80–7.

Rossi G, Barnoud J, Monticelli L. 2014. Polystyrene nanoparticles perturb lipid membranes. J Phys Chem Lett 5:241–6.

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

Sarsour EH, Kalen AL, Goswami PC. 2014. Manganese superoxide dismutase regulates a redox cycle within the cell cycle. Antioxid Redox Signal 20:1618–27.

Sea Urchin Genome Sequencing Consortium. 2006. The genome of the sea urchin Strongylocentrotus purpuratus. Science 314:941–52.

Siller L, Lem Loh ML, Piticharoenphun S, Mendis BG, Horrocks BR, Brümmer F, Medaković D. 2013. Silver nanoparticle toxicity in sea urchin *Paracentrotus lividus*. Environ Pollut 178:498–502.

Snell TW, Hicks DG. 2011. Assessing toxicity of nanoparticles using Brachionus manjavacas (Rotifera). Environ toxicol 26:146–152.

Tamboline CR, Burke RD. 1992. Secondary mesenchyme of the sea urchin embryo: ontogeny of blastocoelar cells. J Exp Zool 262:51–60.

Torres-Duarte C, Adeleye AS, Pokhrel S, Mädler L, Keller AA, Cherr GN. 2016. Developmental effects of two different copper oxide nanomaterials in sea urchin (Lytechinus pictus) embryos. Nanotoxicology 10:671–9.

Tsai YP, Teng SC, Wu KJ. 2008. Direct regulation of HSP60 expression by c-MYC induces transformation. FEBS Lett 582:4083–8.

Voellmy R. 2004. On mechanisms that control heat shock transcription factor activity in metazoan cells. Cell Stress Chaperones 9:122–33.

Wang F, Bexiga MG, Anguissola S, Boya P, Simpson JC, Salvati A, Dawson KA. 2013. Time resolved study of cell death mechanisms induced by amine-modified polystyrene nanoparticles. Nanoscale 5:10868–76.

Ward JE, Kach DJ. 2009. Marine aggregates facilitate ingestion of nanoparticles by suspension-feeding bivalves. Mar Environ Res68:137–42.

Wegner A, Besseling E, Foekema EM, Kamermans P, Koelmans AA. 2012. Effects of nanopolystyrene on the feeding behavior of the blue mussel (Mytilus edulis L.). Environ Toxicol Chem SETAC 31:2490–7.

Wright SL, Thompson RC, Galloway TS. 2013. The physical impacts of microplastics on marine organisms: a review. Environ Pollut 178:483–92.

Wu B, Torres-Duarte C, Cole BJ, Cherr GN. 2015. Copper oxide and zinc oxide nanomaterials act as inhibitors of multidrug resistance transport in sea urchin embryos: their role as chemosensitizers. Environ Sci Technol 49:5760–70.

Xia T, Kovochich M, Brant J, Hotze M, Sempf J, Oberley T, et al. 2006. Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm. Nano Lett 6:1794–807.

Zhang H, Kuo Y-Y, Gerecke AC, Wang J. 2012. Co-release of hexabromocyclododecane (hbcd) and nano- and microparticles from thermal cutting of polystyrene foams. Environ Sci Technol 46:10990–6.

Zhu H, Qiu H, Yoon HW, Huang S, Bunn HF. 1999. Identification of a cytochrome b-type NAD(P)H oxidoreductase ubiquitously expressed in human cells. Proc Natl Acad Sci USA 96:14742–7.

Zito F, Costa C, Sciarrino S, Poma V, Russo R, Angerer LM, Matranga V. 2003. Expression of univin, a TGF-beta growth factor, requires ectoderm-ECM interaction and promotes skeletal growth in the sea urchin embryo. Dev Biol 264:217–27.