Chemosphere 139 (2015) 486–495

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Multidisciplinary screening of toxicity induced by silica nanoparticles during sea urchin development

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HIGHLIGHTS

- Sea urchin fertilization ability was not affected by SiO_2 NPs exposure.
- A significant percentage of anomalies were observed and quantified in exposed samples.
- Altered expression of acetylated tubulin, ChAT and AChE was found in exposed samples.
- The multidisciplinary approach was able to verify SiO_2 NP effects in the offspring.

ARTICLE INFO

Article history:
Received 14 April 2015
Received in revised form 13 July 2015
Accepted 24 July 2015
Available online 24 August 2015

Keywords:
Acetylcholinesterase activity
Developmental alteration
Echinoderms
Ecotoxicology
Nanotoxicity
Sea urchins

ABSTRACT

The aim of this study was to investigate the potential toxicity of Silica nanoparticles (SiO_2 NPs) in seawater by using the sea urchin *Paracentrotus lividus* as biological model. SiO_2 NPs exposure effects were identified on the sperm of the sea urchin through a multidisciplinary approach, combining developmental biology, ecotoxicology, biochemistry, and microscopy analyses. The following responses were measured: (i) percentage of eggs fertilized by exposed sperm; (ii) percentage of anomalies and undeveloped embryos and larvae; (iii) enzyme activity alterations (acetylcholinesterase, AChE) in the early developmental stages, namely gastrula and pluteus. Sperms were exposed to seawater containing SiO_2 NPs suspensions ranging from 0.0001 mg/L to 50 mg/L. Fertilization ability was not affected at any concentration, whereas a significant percentage of anomalies in the offspring were observed and quantified by means of EC_{50} at gastrula stage, including undeveloped and anomalous embryos (EC_{50} = 0.06 mg/L), and at pluteus stage, including skeletal anomalies and delayed larvae (EC_{50} = 0.27 mg/L). Moreover, morphological anomalies were observed in larvae at pluteus stage, by immunolocalizing molecules involved in larval development and neurotoxicity effects – such as acetylated tubulin and choline acetyltransferase (ChAT) – and measuring AChE activity. Exposure of sea urchins to SiO_2 NPs caused neurotoxic damage and a decrease of AChE expression in a non-dose-dependent manner.

In conclusion, through the multidisciplinary approach used in this study SiO_2 NPs toxicity in sea urchin offspring could be assessed. Therefore, the measured responses are suitable for detecting embryo- and larval- toxicity induced by these NPs.

1. Introduction

Nanoparticles (NPs) are ultrafine particles with lengths in two or three dimensions greater than 1 nm and smaller than 100 nm (British Standards Institution, 2005; ASTM, 2006). Today, a broad spectrum of NPs of different chemical composition, size, shape and surface structure are commercially available with several industrial, biotechnological, and biomedical/pharmaceutical applications (Clèment et al., 2013). Among the various types of NPs, silica nanoparticles (SiO_2 NPs) have become popular as nanostructuring, drug delivery, and optical imaging agents (Yang et al., 2010). In particular, they are used in paints and coatings,

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http://dx.doi.org/10.1016/j.chemosphere.2015.07.072
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attachment and scratch-resistance. In printer toners they serve as anti-binder (Mizutani et al., 2006; Zappa et al., 2009). In addition, these NPs are used in chemical or mechanical polishing processes, including dental polishing to prevent tooth caries (Gaikwad and Sokolov, 2008). Other medical applications use SiO_2 NPs as carriers of therapeutic agents or for diagnostic purposes (Wang et al., 2006; Zhang et al., 2008a). While their special physicochemical properties make them commercially attractive, SiO_2 NPs may pose potential hazards to human health and the environment due to their highly stable molecules, which could bioaccumulate in the environment (Nguyen et al., 2013). Since industrial products and domestic wastes tend to end up in waterways, the aquatic environment and biota are of a major concern (Moore, 2006; Baun et al., 2008). Therefore, any direct and/or indirect release of these NPs into the aquatic ecosystem may pose potential hazards to the environment. Indeed, several research works have fully demonstrated that SiO_2 NPs are toxic for freshwater and marine organisms (Van Hoecke et al., 2008; Canesi et al., 2010a,b; Falugi et al., 2012; Gambardella et al., 2013, 2014; Vo et al., 2014). SiO_2 NPs toxicity has already been reported for freshwater algae, namely *Pseudokirchneriella subcapitata*, *Chlorella kessleri* (Fujiwara et al., 2008; Van Hoecke et al., 2008) and for crustaceans, such as *Daphnia magna* (Lee et al., 2009). SiO_2 NPs have been reported to be stable in *P. subcapitata* test medium and that their ecotoxicity was related to their surface area. Further, size-dependent toxicity was reported for SiO_2 NPs in *C. kessleri* and dose-dependent toxicity in crustaceans (Fujiwara et al., 2008; Lee et al., 2009).

Toxicity of SiO_2 NPs has also been reported in the marine environment, caused by unwanted nanosized silica resulting from the release of commercial products, as well as from natural sources, such as coastal erosion, diatom deposits, via rivers to the coastal waters in front of some huge deposits (Buzea et al., 2007). Studies have proved that SiO_2 NPs may induce oxidative stress and evoke significant changes in lysosomal and enzymatic biomarkers in mussels at very high concentrations (up to 10 mg/L, Canesi et al., 2010a, b; Ciacci et al., 2012), apart from affecting algae growth (Zhang et al., 2008b). The uptake, transfer and toxicity associated to SiO_2 NPs from the marine microalgae *Cricosphaera elongata* to sea urchin *Paracentrotus lividus* larvae has recently been investigated. Our research indicates that SiO_2 NPs may enter the marine food chain and, as a consequence, cause abnormalities in developing sea urchin larvae. In addition, a significant survival percentage reduction was observed in sea urchin larvae fed with microalgae previously exposed to 1 mg/L SiO_2 NPs (Gambardella et al., 2014).

P. lividus is a valid model for ecotoxicology studies, and it has been recently proposed as a suitable organism for nanotoxicity studies. Indeed, toxicity tests have reported that its developmental stages are highly sensitive to several NPs (Fairbairn et al., 2011; Manno et al., 2012; Matranga and Corsi, 2012; Manzo et al., 2013; Siller et al., 2013). Due to their sensitivity and availability, *P. lividus* gametes are usually employed to assess toxicity of chemical compounds and NPs in particular. Therefore, spermiotoxicity tests may help study the effects evoked by NPs on developing sea urchin larvae. Sperm cells can also result in transmissible damage to the offspring when exposed to NPs (Gambardella et al., 2013; Manzo et al., 2013; Mesarič et al., 2015). However, there are no data yet about their toxic effects on *P. lividus* embryos and larvae from sperms exposed to SiO_2 NPs.

In this study, the effects of exposure to SiO_2 NPs were identified on the sperm of the sea urchin, through a multidisciplinary approach, combining developmental biology, ecotoxicology, biochemistry, and microscopy analyses. In particular, the following responses were measured: (i) percentage of fertilized eggs by exposed sperm; (ii) percentage of anomalies and undeveloped embryos and larvae; (iii) enzyme activity (acetylcholinesterase, AChE, E.C. 3.1.1.7) in the early developmental stages. Moreover, morphological anomalies were investigated in larvae at pluteus stage, by immunolocalizing molecules involved in neurotoxicity activity and larval development, such as acetylated tubulin and choline acetyltransferase (ChAT, E.C. 2.3.1.6).

2. Materials and methods

2.1. Nanoparticles

SiO_2 NPs with a nominal size between 4 and 40 nm were provided by Tal Materials Inc (USA) with at least 98% purity. NPs were characterized for size and effective surface charge (ζ-potential). Prior to each measurement, SiO_2 NPs were resuspended in ultrafiltered (0.22 μm Teflon filter) seawater (FSW, 37‰ salinity) for size characterization and in distilled water for measuring the effective surface charge at a concentration of 50 mg/L, and sonicated for 1 h using Branson 2510 bath sonicator (Branson Ultrasonic, Danbury, CT, USA). Size characterization of NPs dispersed in FSW was determined by Dynamic Light Scattering (DLS) using a Malvern Zetasizer nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Measurements were conducted at 25 °C by transferring 1 mL of stock solution to a square cuvette for DLS analysis. A 50 mW laser with 638.2 nm wavelength was used as light source. Measurements were recorded at a detection angle of 173° (backscatter). The same measurements were also repeated after 1 h, in order to detect any agglomerates in FSW. NPs ζ-potential was measured using a Malvern Zetasizer nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Measurements were conducted at 25 °C by transferring 1 mL of stock solution to a square cuvette for ζ-potential measurements.

2.2. Samples

Mature specimens of *P. lividus* were obtained from the hatchery in Camogli (CNR, Genoa, Italy), where they were maintained in ultrafiltered seawater (FSW). Adult samples were brought to the laboratory following the method suggested by Amemiya (1996), i.e. in a refrigerated bag, wrapped in tissues wet with sea water.

In this study, a spermiotoxicity test was performed. Therefore, sperms were exposed to NPs and the outcome developmental stages were analyzed. For brevity purposes, we shall refer to the larvae obtained from eggs fertilized by sperm exposed to the NPs as "exposed samples" and refer to sperm exposure with the words "treatment or "exposure".

2.3. Spermiotoxicity test

Spawning of gametes was obtained by oral administration of 1:1000 acetylcholine in FSW to avoid stress or toxicity associated with other current procedures. Eggs were collected in standard FSW, while sperms were collected 'dry', directly from the genital pores and maintained in aliquots of 200 μl at $T = 4$ °C.

Sperm was mixed from 3 different specimens. Experiments were repeated 4 times during breeding season and were carried out in triplicate.

For the test, 10 μl of 'dry' sperm were exposed to SiO_2 NP suspensions at serial concentrations (0.0001, 0.001, 0.01, 0.1, 1, 5, 10, 50 mg/L) for 1 h, by adding 1 mL NP suspension to the eppendorf vials, as previously described in Gambardella et al. (2013). Since to date no environmental concentration in both freshwater and seawater is available either in bibliography or in official database for SiO_2, test concentrations were chosen on the basis of ecotoxicological results of freshwater and marine species exposed to SiO_2 NPs, that tested concentrations up to 100 mg/L (Adams

et al., 2006; Canesi et al., 2010a,b; Ciacci et al., 2012; Casado et al., 2013; Gambardella et al., 2014). Negative controls were performed by adding 1 mL of standard FSW. After 1 h, 50 µL of sperm from each vial were added to multiwell capsules containing approximately 300 eggs/ml in 10 mL standard FSW. The reliability of the test was verified using the reference toxicant copper nitrate as positive control, according to Arizzi Novelli et al. (2002). The acceptability of the results was fixed at a fertilization rate and percentage of normal development >70% in control tests (Volpi Ghirardini and Arizzi Novelli, 2001; Arizzi Novelli et al., 2002).

Egg activation occurs within 60 s of the addition of sperm as previously reported (Gambardella et al., 2013). The test was performed following the methods suggested by Volpi Ghirardini et al. (2005): i.e., 2 mL of water containing approximately 600 zygotes were collected from each well 20 min after sperm addition and fixed by 4% paraformaldehyde in FSW. Samples were observed under a light microscope (DM3000B, Leica, Germany) at 40× magnification. Eggs with elevation of the fertilization membrane and swollen perivitelline space were monitored and the Effective Concentration resulting in 50% fertilization inhibition in the exposed organisms (EC_{50}) calculated.

2.4. Embryo- and larval-toxicity test

The remaining zygotes were left to develop at 18 °C in a thermostatic room and part of them were sampled at 24 and 72 h, corresponding to gastrula and pluteus stage, respectively. These stages were then fixed in 2% paraformaldehyde in FSW and permeabilized by cold methanol and 20% polyethylene glycol (Sigma, Italy). Fixed embryos and larvae were rehydrated in 0.1% Bovine Serum Albumine (BSA)/Phosphate Buffered Saline (PBS, pH 7.4) for 10 min before further processing for microscopic observations and immunohistochemical reactions. Approximately 100 embryos and larvae were mounted on slides and their characteristics compared between different concentrations of SiO_2 NPs and controls.

The state of the embryos and larvae was checked under a Leica light microscope (DM3000B, Leica, Germany) and classified by morphology and synchronicity of development as compared with controls. In detail, gastrulae were separated into three categories, designated as developed (D), anomalously developed (AD) and non-developed embryos (ND), according to Gambardella et al. (2013). D embryos showed normal development, with well structured archenteron and migratory cells ingressed into the coeloma; ND embryos referred to both arrested development of embryos and gastrulae lacking archenteron and coeloma; AD embryos referred to defective gastrulae, with typical signs of asymmetrical migration of primary mesenchyme cells. These anomalies were observed and quantified by means of EC_{50} (Effective Concentration resulting in 50% developmental anomalies in the exposed organisms) at gastrula stage, including ND and AD embryos. ND embryos were discarded after 24 h stage, when larvae swimming in the water column were selected and used for analyzing pluteus development. Plutei were then separated into two categories: delayed larvae (DEL) and larvae with skeletal (SK) anomalies, according to Roepke et al. (2005). Delayed larvae were plutei which appeared to have normally developed, but failed to reach the same stage as controls. Skeletal anomalies comprized larvae with misshapen spicules, missing arms, elongated larvae. These anomalies were observed and quantified by means of EC_{50} at pluteus stage.

2.5. Immunohistochemical stainings

Part of the 72 h larvae derived from 1 mg/L exposed sperms were used to detect morphological anomalies, by immunolocalization of acetylated tubulin and ChAT, as well as to measure ChE activity. Owing to the high percentage of anomalies (>80%), plutei derived from sperms exposed from 5 mg/L up to 50 mg/L were discarded for immunohistochemical and ChE activity analyses, for the overall outcome would be biased by further analyses on these samples.

Plutei were incubated overnight at 4 °C with anti-acetylated tubulin antibody (1:300, Sigma Chemical Co, USA) raised in mouse and anti-ChAT antibody (1:400, Chemicon, USA) raised in rabbit, diluted in PBS containing 0.5% BSA, 0.1% Normal Goat Serum (NGS). Nuclei were counterstained with 1 µM RNAase followed by 2 µM propidium iodide (PI). After three washes in 1% BSA in PBS for 10 min each, sections were incubated for 2 h at room temperature (25 °C) with appropriate secondary anti-rabbit and anti-mouse antibody (1:300 in PBS) conjugated with Alexa Fluor 488. After rinsing in PBS, samples were mounted in gelvatol and about 50 plutei were observed under a Confocal Laser Scanning Microscope (CLSM microscope).

2.6. Image acquisition and analysis

Slices and 3D images (1024 × 1024 × 8 bit) were acquired using a Leica TCS SP5 AOBS CLSM microscope (Leica Microsystems, Mannheim, Germany) with a one Airy disk unit pinhole diameter and an HCX PL APO 63 × 1.4 oil immersion objective. Magnifications were obtained by scan field selection. Alexa Fluor 488 was excited with the 488 nm line of an Argon laser. Dye fluorescence was collected in a 500–530 nm spectral window. PI was excited with the 543 line of the He-Ne laser, and its fluorescence was collected in a spectral 600–640 nm window. Laser scanning transmitted light images were obtained using the 488 nm laser line.

2.7. Acetylcholinesterase activity

AChE activity was measured by Ellmann's (1961) method, which was modified *ad hoc* for the Jenway (6405 UV/vis) spectrophotometer. 72 h development larvae were collected and maintained for 2 weeks at −20 °C and then homogenated and sonicated for 25 min in a bath sonicator (FALC, mod. LBS1, Italy). The larvae were passed through a very thin syringe needle (Ultrafin 29G, 12,7 mm length) in the presence of 1% triton X100, and centrifuged for 30 s at 8000 rpm. AChE activity kinetics was measured by $\lambda = 412$ nm absorbance. Substrate cleavage speed was measured for 3 min and compared with the linear equation of a standard curve previously obtained by supplying known amounts of AChE. Protein content in control supernatants and in exposed plutei was measured according to the Bradford method, using Biorad reagent, and referred to a standard curve obtained from albumin use (BSA, Sigma). AChE units were obtained by calculating the ratio between enzyme activity/min/mg protein. All measurements were performed with plutei from different parents, in triplicate, and were repeated four times during *P. lividus* reproductive season.

As previously reported, we shall refer to the larvae obtained from eggs fertilized by sperm exposed to the NPs as 'exposed samples' and refer to sperm exposure with the words 'treatment' or 'exposure'.

2.8. Statistical data analysis

All experiments were carried out in triplicate. All data are expressed as means ± standard error (SE) of the triplicates. EC_{50}s and related 95% confidence limits were calculated using the Probit method. Significant differences between controls and treated samples were determined using one-way ANOVA followed by the Bonferroni nonparametric *post hoc* tests.

3. Results

3.1. Nanoparticle characterization

The results of NP characterization are summarized in Table 1, reporting size and ζ-potential of SiO_2 NPs. The characterization, especially under the conditions in which the NPs will be presented to the sperm (in FSW, 37‰ salinity for 1 h), is essential in order to understand the dose of NPs that is being presented to the sperm. Results show that SiO_2 NP size increased after 1 h of exposure in both solutions diluted in FSW and water. These data highlight a key limitation of the DLS technique, that it gives a mean value, which is physically quite meaningless in the presence of multiple peaks such as are observed here. However, for the purpose of our study, the understanding of the NP solutions in FSW over time was necessary and the DLS analysis showed a shift in apparent particle size after 1 h, suggesting an increase of agglomeration. The ζ-potential was negative in SiO_2 NPs diluted in distilled water.

3.2. Spermiotoxicity

Results on spermiotoxicity tests are reported in Fig. 1. Controls showed <5% of unfertilized eggs and the repeatability of the experiments was tested by using copper nitrate as reference toxicant. The test had a good repeatability, with an egg fertilization decrease of 53 ± 2% at 0.01 mg/L (not shown), similar to previous results reported by Manzo et al. (2008). Fertilizing ability did not appear to be affected by SiO_2 NPs at any concentrations. At the highest concentration (50 mg/L), 13.3% ± 2.4 of the eggs failed to exhibit an elevated fertilization layer 40 min after sperm addition, therefore the EC_{50} could not be calculated. No NP agglomerates were observed in FSW, due to short-term exposure (1 h).

3.3. Embryotoxicity

The percentage of developed (D), anomalous (AD) and non developed (ND) embryos sampled at 24 h after fertilization is shown in Fig. 2A. The percentage of D embryos gradually decreased, being NP-dose dependent and statistically different from controls ($p < 0.01$). Conversely, the percentage of AD embryos was not NP-dose dependent: the highest percentage of anomalies, including defective gastrulae, were observed at the lowest tested NP-concentration (0.0001 mg/L), while < 20% of anomalies were found at other concentrations. AD embryos were significantly different from controls at low concentrations ($p < 0.01$ mg/L).

A high percentage of ND gastrulae statistically different from controls ($p < 0.01$) was observed. This percentage increased up to 70% in a non-dose-dependent manner in embryos from sperms exposed to 50 mg/L SiO_2 NPs. This concentration was significantly different from the others ($p < 0.05$). EC_{50} (0.06 mg/L [confidence limits 0.01–0.44]) could be calculated from the percentage of non-developed embryos (ND) and anomalous embryos (AD) taken together.

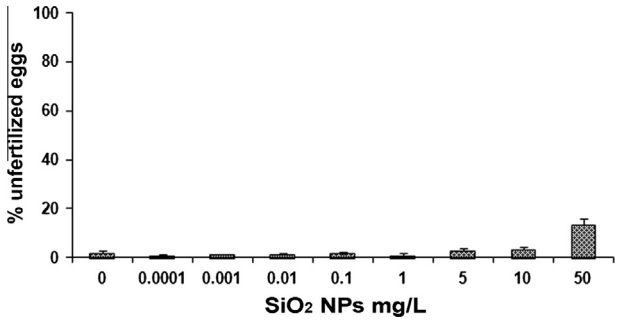

Fig. 1. Percentage of unfertilized *P. lividus* eggs after addition of sperm exposed to different SiO_2 NPs concentrations.

3.4. Larval toxicity

Larvae sampled at 72 h, corresponding to pluteus stage of controls, had a high percentage of anomalies, ranging from 35% to 100% (Fig. 2B). All anomalies (SK and DEL) were significantly different from the controls ($p < 0.01$) and the percentage of larvae was NP-dose dependent. The EC_{50} (0.27 mg/L [confidence limits 0.09–0.87]) could be calculated from the percentage of DEL and SK.

Abnormal plutei were counted separately according to recorded defects, which were classified as delayed larvae (DEL) or plutei bearing skeletal anomalies (SK), such as irregular skeletal spines and incomplete skeletal rods (Fig. 3). Main anomalies included larvae showing crossed skeletal tips at the hood apex (Fig. 3b), plutei with joined anterior arms (Fig. 3c), plutei with naked tip of the skeletal spine (Fig. 3d), supernumerary skeletal rods (Fig. 3e) and delayed larva with the development stopped at gastrula stage (Fig. 3f and g).

3.5. Immunohistochemical localization of acetylated tubulin and ChAT

To investigate SiO_2 NPs effects at cellular level, acetylated tubulin-staining and ChAT staining were used to monitor larval development and neurotoxicity activity during pluteus stage. Acetylated tubulin-immunostaining is shown in Fig. 4, where control plutei are shown with numerous strongly stained cilia, mostly all over the larvae surface and in the stomach (Fig. 4a–c). Less fluorescent cilia were observed in the surface of treated larvae exposed to 0.0001 mg/L SiO_2 NPs (Fig. 4d–f) and fully disappeared in those from sperms exposed to the highest SiO_2 NPs concentrations (Fig. 4g and h). At this concentration, also cilia appeared to be severely damaged (Fig. 4i and j).

ChAT immunostaining is shown in Fig. 5, where immunoreactivity was observed in ganglia lateral to the esophagus and on the surface of control plutei, corresponding to the basal bodies of the cilia (Fig. 5a). A similar distribution was found in exposed samples (Fig. 5b–e), where fluorescence in the stomach and perioral body surface seemed to decrease with higher SiO_2 NPs concentrations (e.g. after exposure to 0.01 and 1 mg/L SiO_2 NPs, Fig. 5d and e).

Table 1
Physicochemical characterization of SiO_2 NPs. NP size and ζ-potential analysis were prepared in distilled water (H_2O) and FSW. Due to high poly-dispersity index (PDI), the values and percentage of peak 1 and peak 2 are reported. DLS and ζ-potential results are the average of a minimum of three separate runs; errors represent the standard deviation over measurements and are intended solely as an indication of the reproducibility of the measurement.

Sample	Solution	*T* (°C)	Diameter[a] (nm)	PDI[b]	Peak 1	Peak 2	ζ-potential (mV)
SiO_2 NPs	H_2O	25	275	0.30	264 ± 89 (99%)	5429 ± 287 (1%)	−38 ± 12
SiO_2 NPs	FSW	25	965	0.25	890 ± 238 (99%)	5500 ± 204 (1%)	–
SiO_2 NPs	H_2O-1 h	25	277	0.31	268 ± 91 (97%)	5209 ± 469 (3%)	−38 ± 13
SiO_2 NPs	FSW-1 h	25	1204	0.28	1370 ± 274 (67%)	613 ± 88 (33%)	–

[a] z-average hydrodynamic diameter extracted by cumulant analysis of the data.
[b] Polydispersity index from cumulant fitting of the data.

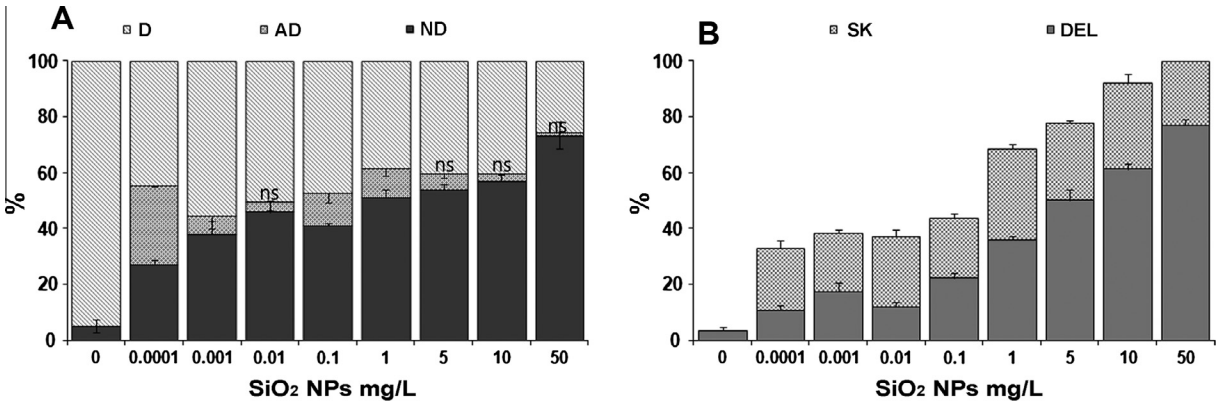

Fig. 2. A: *P. lividus* gastrulae at 24 h after fertilization with sperm exposed to different SiO_2 NPs concentrations. Percentages are compared to controls. ND = no development; AD = anomalous development; D = normal development. ns = not significant differences in anomalous development of treated embryos compared to controls ($p > 0.05$). B: *P. lividus* plutei at 72 h after fertilization with sperm exposed to different SiO_2 NPs concentrations. Percentage of anomalies in plutei at 72 h development: DEL = delayed larvae; SK = skeletal anomalies.

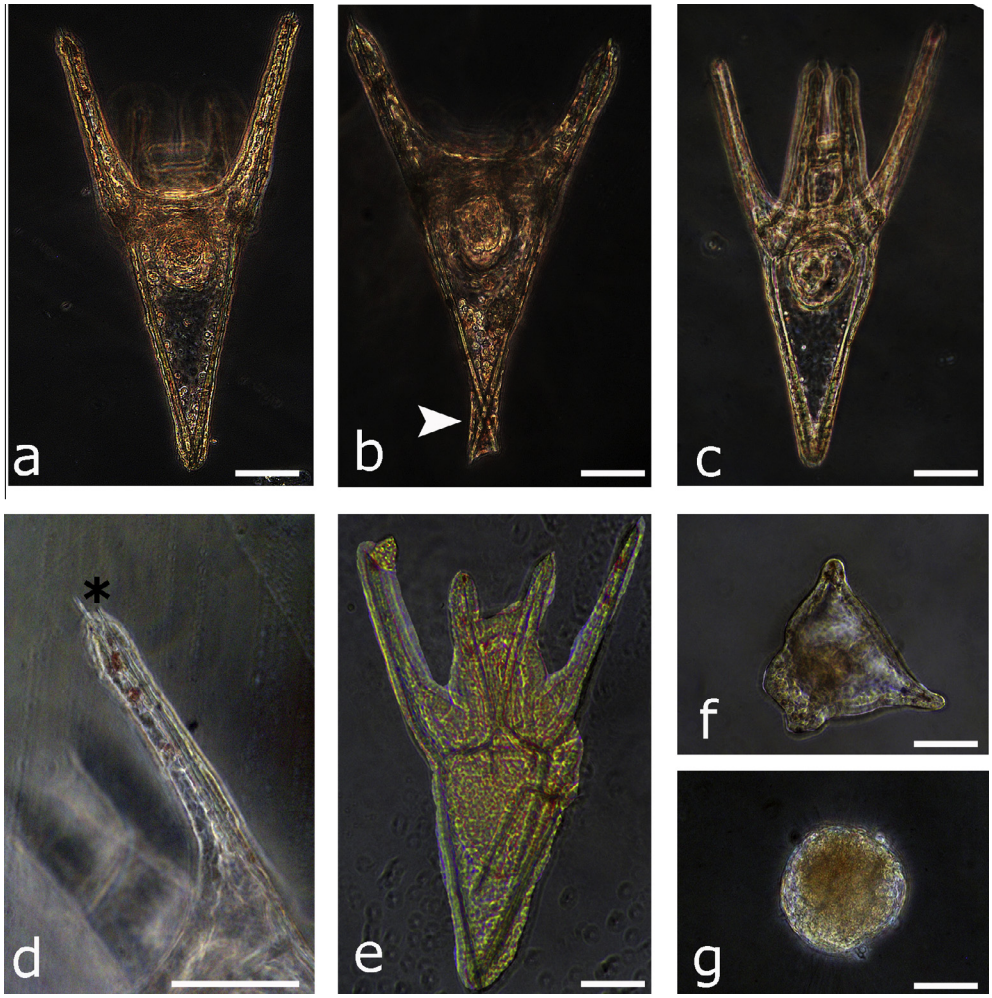

Fig. 3. Main anomalies found at *P. lividus* pluteus stage. (a) Pluteus control showing correct position of skeletal rods; (b) pluteus showing crossed skeletal tips at the hood apex (arrowhead, 0.0001 mg/L SiO_2 NPs); (c) pluteus with joined anterior arms (1 mg/L SiO_2 NPs); (d) asterisk showing naked tip of the skeletal spine in 5 mg/L SiO_2 NPs treated-pluteus; (e) supernumerary skeletal rods and joined anterior perioral arms (10 mg/L SiO_2 NPs); (f and g) delayed larva: development stopped at gastrula stage (50 mg/L SiO_2 NPs). Bar equals 100 μm.

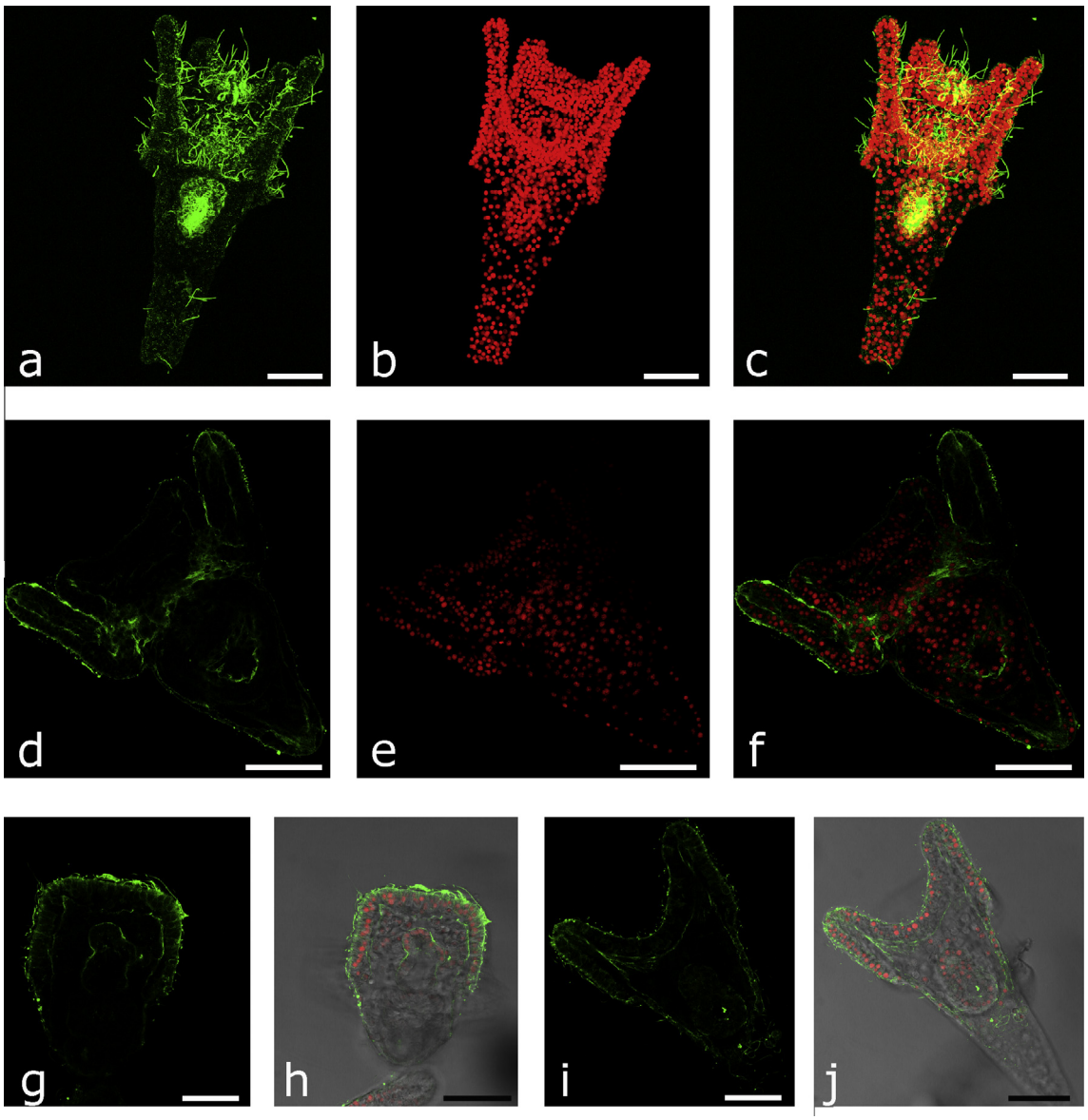

Fig. 4. Confocal Laser Scanning Microscopy images of acetylated tubulin immunolocalization at *P. lividus* pluteus stage. (a–c) Pluteus control with acetylated tubulin staining in the stomach and on cilia surface; (d–f) anomalous pluteus with weak immunostaining at stomach and arm level (0.0001 mg/L SiO_2 NPs); (g and h) delayed larvae with acetylated tubulin immunostaining on cilia surface (1 mg/L SiO_2 NPs); (i and j) immunostaining of pluteus surface cilia (0.1 mg/L SiO_2 NPs). Nuclei are counterstained with PI (red). Bar equals 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Cholinesterase activity

AChE activity could be measured in all samples (Fig. 6). AChE was affected by SiO_2 NPs in the larvae obtained from exposed sperm. In general, AChE activity was impaired by each suspension of SiO_2 NPs, and a significant difference was observed between controls and exposed larvae ($p < 0.01$). The effects did not appear to be dose-dependent, with only insignificant differences among samples exposed to various concentrations ($p > 0.05$).

4. Discussion

4.1. SiO_2 NP characterization

We reported the chemical characterization of SiO_2 NP suspension in seawater. The nature of NPs is modified by adsorption processes and especially the surface charge plays a dominant role (Kallay and Zalac, 2002; Fukushi and Sato, 2005). Here, the effective surface charge highlights the negative ζ – potential of SiO_2 NPs, according to previous studies of the same NPs (Yu et al., 2011; Mc Carthy et al., 2012). This result could have influenced the toxicity of such NPs on sea urchin sperms, since the negative charge of the NPs and the hydrophobic sperm cell membrane may be responsible for a weak binding affinity. In addition, other parameters may play a role in reducing sperm toxicity, such as the aggregation process of SiO_2 NPs in seawater. It is known that NPs tend to aggregate in seawater (Canesi et al., 2010a; Kadara et al., 2014) and the formation of agglomerates may protect marine organisms from NP toxicity (Canesi et al., 2012). Therefore, as previously reported in literature, both the NP negative charge and aggregation might lead to a low sperm cell interaction and poor NP internalization, resulting in reduced toxicity (Albanese and Chan, 2011; Fröhlich, 2012). Although we found a reduced toxicity in sea urchin sperm, we observed toxic effect in sea urchin developmental stages from exposed sperm, probably due to the

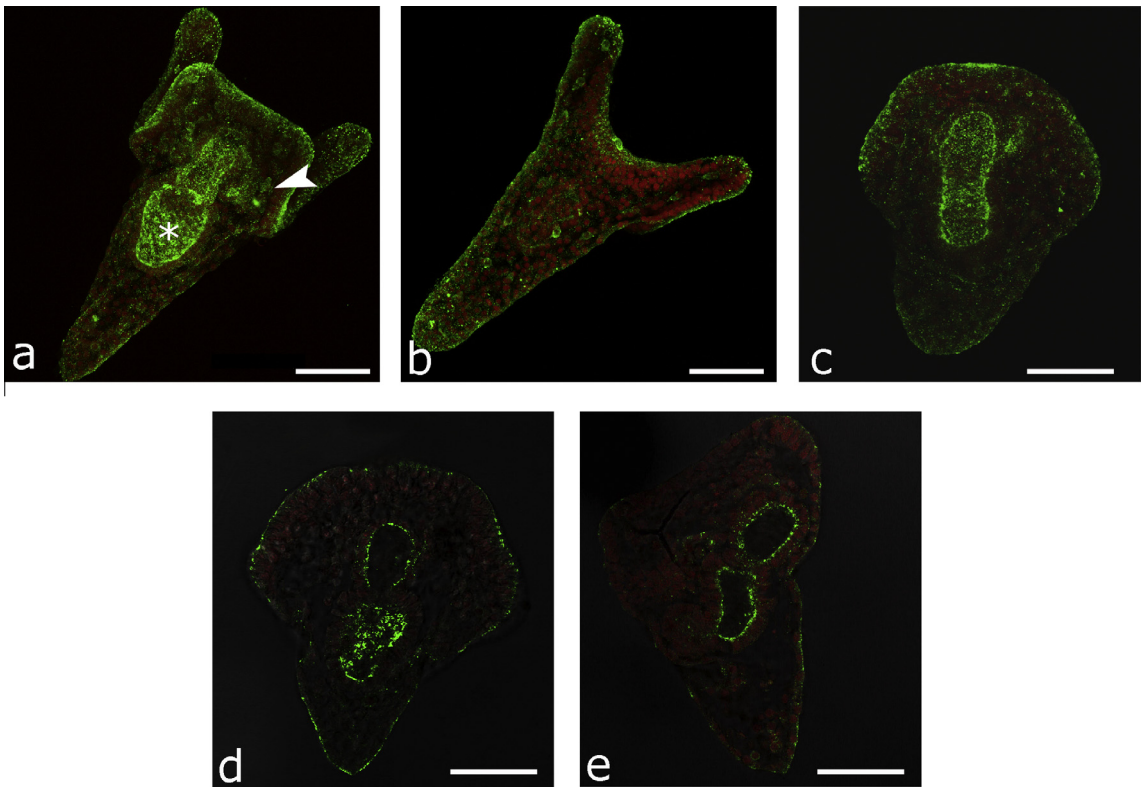

Fig. 5. Confocal Laser Scanning Microscopy images of immunolocalization of ChAT immunolocalization at *P. lividus* pluteus stage. (a) Pluteus control with staining of lateral ganglia (arrowhead), the esophagus and stomach walls (asterisk), and on larvae surface; (b) anomalous pluteus (0.0001 mg/L SiO_2 NPs) with only anterior pair of arms; (c) larva obtained from exposure of sperms to 0.001 mg/L SiO_2 NPs; (d and e) anomalous larvae exposed to 0.01 (d) and 1 mg/L SiO_2 NPs (e), with weak immunostaining of stomach and perioral body surface. Bar equals 50 μm.

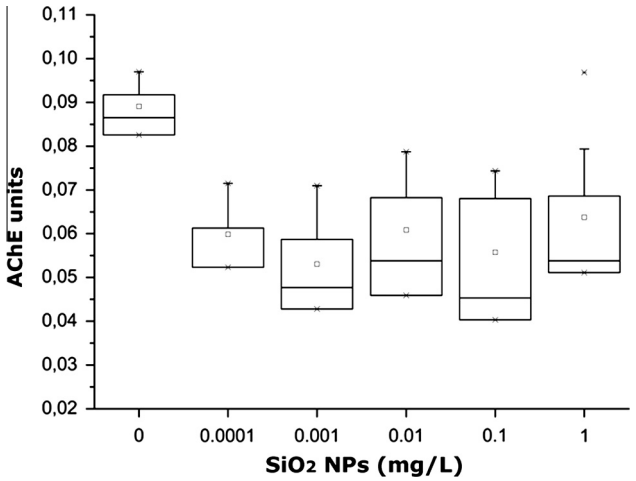

Fig. 6. Effect of exposure to SiO_2 NPs on ChE activity of *P. lividus* sea urchin larvae. All measurements were significantly different from controls ($p < 0.01$).

nanoscale nature of SiO_2 particles. This hypothesis is supported by studies demonstrating that the repulsive energy barriers between metal oxide NPs become negligible when aggregation takes place, because the electrolytes present in seawater saturate the surface negative charge (Zhang et al., 2008a,b).

4.2. Spermiotoxicity

This study aimed at investigating any toxic effects induced by SiO_2 NPs during *P. lividus* development. Since to date no predicted environmental concentration (PEC) of SiO_2 NPs in seawater is available in literature, very high concentrations were employed in this study in order to detect a possible toxic effect, quantified by means of EC_{50}s. Although these concentrations were much higher than would be expected in the environment based on model predictions of other NP release from consumer products (Gottschalk et al., 2009), we demonstrated that SiO_2 NPs did not affect fertilization capability up to 50 mg/L (EC_{50} not calculable), probably due to the chemical nature of such NPs (as previously described in Section 4.1.) but it induced toxic effects on the offspring. These results are consistent with previous findings on sea urchin sperms exposed to other NPs, where TiO_2, ZnO, Ag, Graphene Oxide and Co NPs did not affect sea urchin sperm fertilization capability (Gambardella et al., 2013; Manzo et al., 2013; Mesarič et al., 2015).

4.3. Embryo- and larval-toxicity

4.3.1. Gamete-NP interactions

Short exposure of the sperm in SiO_2 NPs suspensions (1 h) significantly affected offspring development at different organization levels. When NPs are highly concentrated, they may form micrometer-sized particles that can modify the surface properties and the influence of particle size and shape on their toxicity (Handy et al., 2008). These aggregates may be responsible for reducing the toxicity of such NPs. Nevertheless, NP internalization in sea urchin zygote may occur according to Yu et al. (2014). These authors report SiO_2 NP internalization by TEM evidence either as free or as membrane-bound aggregates in the cytoplasm.

Recent studies on sea urchin sperm exposed to other types of NPs demonstrate that although sperm motility and functionality was not affected, the sperms were able to transfer toxic outcomes to the eggs and offspring (Gambardella et al., 2013, 2015a). The

effects we found in sea urchin gastrula and plutei from exposed sperm may be beneficial to enter the zygote rapidly during fertilization. Moreover, considering the negative charge, SiO_2 NPs may need a carrier in order to be transferred inside the cells through, for example, endocytic processes (e.g. clathrin-coated vesicles, Gambardella et al., 2015a).

We may hypothesize the internalization of NPs transported by the sperms inside the zygote, taking into account the previously described literature and the electrical events occurring during fertilization. Actually, fertilization is driven by dramatic electrical events, such as sperm capacitation, a maturation process that induce intracellular changes (e.g. Ca^{2+} increase) and changes in swimming patterns (Breitbart, 2002).

In the capacitated spermatozoon, binding of the sperm to the egg membrane activates a selective cation channel, leading to membrane depolarization and an increase in Ca^{2+} ions. Therefore, when fertilization occurs, SiO_2 NP exposed sperms crowded around the eggs undergo the acrosomal reaction (Gambardella et al., 2015b). When the first sperm touches the inner egg membrane, an explosive release of Ca^{2+} ions from the inner stores occurs. This prevents polyspermy, because the strong positive ion charge repulses the supernumerary sperms crowding around. This is the so-called fast block to polyspermy and lasts some seconds, followed by Ca^{2+}-induced cortical reaction. In these seconds, the highly positively charged zygote is able to attract the NPs present at the sperm surface with strength major than the sperm one. During the cortical reaction and immediately after, pinocytotic vesicles are present at the egg surface, for the recovery of enzymes and residuals. Possibly in this phase the NPs in the perivitelline space and adherent to the membrane may be introduced into the cytoplasm together with the membrane. Thus, the presence of NPs inside the organelles of the zygote may produce inflammation and consequent biochemical outcomes observed by the different authors in marine invertebrates (Canesi et al., 2010a,b, Ciacci et al., 2012).

4.3.2. Effects of SiO_2 NPs on embryonic and larval development

The early development stages of sea urchins are known to be very sensitive, since any contact with toxic substances (e.g. metals, pesticides, NPs, etc.) could lead to major biochemical and physiological changes (Radenac et al., 2001; Caplat et al., 2010; Carata et al., 2012). Thus, changes in embryo and larvae morphology were observed and quantified. We found that the exposure of sea urchin sperm to SiO_2 NPs caused irreversible developmental anomalies in the offspring, affecting both embryonic and larval morphology. Delayed/arrested development, defective and skeletal anomalies are the most common parameters used for detecting toxicity induced by NPs in the early developmental stages of different sea urchin species (Fairbairn et al., 2011; Manzo et al., 2013). These criteria provide very sensitive tools for environmental toxicity assessment (Carballeira et al., 2012). Development modifications of sea urchin embryos and larvae feature phenomenological changes (e.g. amount of anomalies) and the rate of development (e.g. retarded/accelerated progression through developmental stages, Manno et al. 2012, 2013)). A high percentage of undeveloped and anomalous embryos and larvae was observed compared to controls, in line with reports from other authors (Fairbairn et al., 2011; Gambardella et al., 2013; Manzo et al., 2013). Arrested development observed in sea urchins at the gastrula stage increased in a not-dose-dependent manner, mostly resulting from altered energy budgets under abiotic stress regimes (Stumpp et al., 2011). Likewise, anomalies found at gastrula stage were not directly related to SiO_2 NPs amount: more effects were recorded with lower than higher doses. This trend was not observed in the next developmental stage (pluteus), where these anomalies, featuring skeletal anomalies and delayed larvae, occurred in a dose-dependent manner, according to our previous findings on other metal oxide NPs (Gambardella et al., 2013). This may be due to the appearance of anomalies still hidden in earlier stages. As a matter of fact, development is a multiphase event, whereby anomalies not yet observable during early stages are amplified in later ones. These data are indicative of complex interactions between the different sea urchin developmental stages and NPs, which need to be further investigated.

Based on the percentage of undeveloped/delayed and anomalous embryos/larvae, EC_{50}s values were calculated at both gastrula and pluteus stages, following SiO_2 NPs effects. Despite quite different absolute values (0.06 mg/L at gastrula stage versus 0.27 at pluteus stage), the range of effects as indicated by confidence intervals did not differ in the various investigated stages. Therefore, at gastrula and pluteus stage similar sensitivity to SiO_2 NPs was observed, as they were both significantly different from the controls at all concentrations.

4.4. Acetylated tubulin and ChAT localization

Besides morphological changes in treated plutei, different distribution patterns and/or expression of acetylated tubulin and ChAT were also observed. Acetylated tubulin is a molecule involved in larval development. It is found in the microtubules that make up cilia axonemes. In sea urchin larvae, cilia are important for motility and food capture (Emlet, 2002; Hara et al., 2003). Decreased acetylated tubulin immunostaining was observed in the stomach and cilia at the surface of treated plutei, followed by ciliary damage. Changed acetylated tubulin patterns may be related to the stability of microtubules (Palazzo et al., 2003). In our case, this alteration may further shorten the life of acetylated microtubules and hence of the larvae, since they will not be able to feed.

ChAT distribution patterns were not affected in larvae from sperms exposed to SiO_2 NPs. However, this molecule was down-regulated in treated samples compared to controls. ChAT is the biosynthetic enzyme of acetylcholine (ACh), the cholinergic signal molecule (Yasuyama and Salvaterra, 1999; Gallus et al., 2006). It is thus considered to be an excellent marker for cholinergic neurons. ChAT activity is regulated by its binding to the cholinergic membrane (Dobransky and Rylett, 2003; Angelini et al., 2004) and by the interaction with other cellular proteins related to this membrane (Gabrielle et al., 2003). Altered levels of ChAT or ACh might be an inflammatory process reflection (Ratcliffe et al., 1998). Thus, ChAT down-expression could be indicative of decreased ACh production in treated samples (Lv et al., 2014). Therefore, neurotoxic effects seem to be induced by SiO_2 NPs in sea urchin plutei.

4.5. AChE expression

ChAT findings agree with AChE results, showing inhibited AChE activity in treated samples. AChE is the biolytic enzyme for ACh at neuromuscular synapses (Jennings et al., 2008). AChE inhibition is generally responsible for a higher number of uncleaved ACh, justifying poor ChAT immunoreactivity in treated samples, as compared to controls.

Besides its neuromuscular function, AChE has been demonstrated to be responsible for driving apoptosis (Zhang et al., 2002; Park et al., 2004; Toiber et al., 2008; Zhu et al., 2008) and all morphogenetic movements during sea urchin development (Drews, 1975). This could also explain delayed arm elongation in treated plutei likely to be due to skeletogenic cell migration (as shown in Fig. 4).

We observed that AChE activity was impaired by all SiO_2 NPs suspensions and that this effect was not dose-dependent. Previous studies had reported ChE inhibition in sea urchin larvae obtained from sperms exposed to several NPs (Gambardella

et al., 2013; Mesarič et al., 2015) and in sea urchin immune cells associated with the uptake of metal oxide NPs (Falugi et al., 2012). In this study we found a non-dose-dependent response, as demonstrated by statistical analysis. However, a significant difference ($p < 0.01$) was reported between exposed samples and controls, thus confirming that the observed effects depended on NPs exposure.

4.6. Interaction between SiO_2 NPs and marine organisms

Through a multidisciplinary approach, SiO_2 NPs were shown to have toxic effects only on sea urchin offspring and not on their fertilization. These NPs have so far been poorly investigated in marine organisms, while many studies refer to humans (Mc Carthy et al., 2012). To date, SiO_2 NP toxicity has been only demonstrated in marine algae and mussels. High concentrations of SiO_2 NPs inhibit *Dunaliella tertiolecta* growth (EC_{50} = 185.7 mg/L, Manzo et al., 2015) and induce enzyme changes and oxidative stress on mussels (Koehler et al., 2008; Canesi et al., 2010a,b; Ciacci et al., 2012). The mechanism of NPs on organismic health is represented by the rising of Reactive Oxygen Species (ROS) and inflammation (Falugi and Aluigi, 2012), that might lead to enhance the toxicity. ROS-related inflammation has been reported in *Mytilus galloprovincialis* after SiO_2 NP exposure, where it seems to be mediated by rapid activation of the stress-activated p38 (Canesi et al., 2010b). Although no studies on the effects of these NPs on the early developmental stages of marine organisms are yet available, we believe that the decrease of AChE expression in sea urchin larvae obtained from sperms exposed to SiO_2 NPs may be responsible for increasing inflammation by ACh. This hypothesis is supported by previous studies on sea urchin immune cells exposed to other NPs, where the mechanism of action of the NPs was represented by a depression of the immune system, due to ChE activity and molecule expression (i.e. HSP70, GRP78, Falugi et al., 2012). On this basis, the inflammation raises the release of Ach, driving cell proliferation, and causing an indirect effect on AChE activity (Pavlov et al., 2003).

5. Conclusions

In this study, potential SiO_2 NPs toxicity in seawater was investigated by using the sea urchin as model organism.

Many sandy/rocky or muddy bottoms are affected by a massive release of SiO_2 NPs into coastal waters, from both anthropic and natural sources (e.g. commercial products, mining, coastal erosion, etc.; Buzea et al., 2007). SiO_2 NPs intake by sea urchins occurs through grazing of the substrate and of the above-lying biofilm, or eating contaminated algae, as previously demonstrated (Gambardella et al., 2014). Once ingested by sea urchins, SiO_2 NPs may enter their coelomic fluid and move to different tissues, including gonads (Falugi et al., 2012), where they reach the gametes. This process may explain why SiO_2 NPs could interfere with the reproduction success of benthic organisms feeding on the substrate. Transfer of NPs effects to planktonic offspring may significantly affect the biodiversity of contaminated sites. With our multidisciplinary approach we have obtained morphological, biochemical, molecular and ecotoxicological information, which, at least in part, has allowed us to identify the mechanisms of SiO_2 NPs toxicity in the early development of benthonic marine organisms and the transfer of its effects onto the offspring. With our integrated study, the effects induced by SiO_2 NPs in sea urchin embryos and larvae could be detected.

Acknowledgments

This work has been supported by RITMARE (Ricerca Italiana per il MARE) Flagship Project, a National Research Programme funded by the Italian Ministry of University and Research (MIUR). Authors also express their sincere thanks to the Nanochemistry Department team of IIT (Istituto Italiano di Tecnologia, Genova, Italy) for providing laboratory facilities for NP physicochemical characterization.

This manuscript has been included in the top 10 list of the 2014 Faranda Prize awards, a commemorative initiative in honour of Francesco Maria Faranda, founder of the CoNISMa, the National Interuniversity Consortium of Universities for Marine Sciences (Rome, Italy).

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