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Effect of silver nanoparticles on Mediterranean sea urchin embryonal development is species specific and depends on moment of first exposure

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Abstract

With the ever growing use of nanoparticles in a broad range of industrial and consumer applications there is increasing likelihood that such nanoparticles will enter the aquatic environment and be transported through freshwater systems, eventually reaching estuarine or marine waters. Due to silver's known antimicrobial properties and widespread use of silver nanoparticles (AgNP), their environmental fate and impact is therefore of particular concern. In this context we have investigated the species-specific effects of low concentrations of 60 nm AgNP on embryonal development in Mediterranean sea urchins *Arbacia lixula*, *Paracentrotus lividus* and *Sphaerechinus granularis*. The sensitivity of urchin embryos was tested by exposing embryos to nanoparticle concentrations in the 1-100 $\mu\text{g L}^{-1}$ range, with times of exposure varying from 30 min to 24 h (1 h to 48 h for *S. granularis*) post-fertilisation which corresponded with fertilized egg, 4 cell, blastula and gastrula development phases. The most sensitive species to AgNP was *A. lixula* with significant modulation of embryonal development at the lowest AgNP concentrations of 1-10 $\mu\text{g L}^{-1}$ with high numbers of malformed embryos or arrested development. The greatest impact on development was noted

for those embryos first exposed to nanoparticles at 6 and 24 h post fertilisation. For *P. lividus*, similar effects were noted at higher concentrations of 50 $\mu\text{g L}^{-1}$ and 100 $\mu\text{g L}^{-1}$ for all times of first exposure. The *S. granularis* embryos indicated a moderate AgNP impact, and significant developmental abnormalities were recorded in the concentration range of 10-50 $\mu\text{g L}^{-1}$. As later post-fertilisation exposure times to AgNP caused greater developmental changes in spite of a shorter total exposure time led us to postulate on additional mechanisms of AgNP toxicity. The results herein indicate that toxic effects of AgNP are species-specific. The moment at which embryos first encounter AgNP is also shown to be an important factor in the development of abnormalities, and future applications of the sea urchin embryo development test for nanoparticle toxicity testing should carefully address the specific phase of development of embryos when nanoparticles are first introduced.

Keywords

silver nanoparticle; sea urchin embryo development test; *Arbacia lixula*; *Paracentrotus lividus*; *Sphaerechinus granularis*

1 Introduction

Engineered nanoparticles (ENP), because of their specific size-related properties are finding increasing use in a broad range of applications in the fields of electronics, biomedicine, agriculture, textiles, chemical, pharmaceutical and food industry, water remediation, personal care products and cosmetics. As a consequence, ENP are increasingly released into the environment, either wittingly or by chance, and eventually arrive in aquatic ecosystems (rivers, lakes, estuaries and coastal areas), thus posing potentially serious risks to those environmental niches. Therefore, investigating the potential for ENP toxicity to living organisms, albeit somewhat belatedly for coastal areas, has become an important issue (Baker *et al.*, 2014; Corsi *et al.*, 2014; Matranga and Corsi, 2012). In particular, the main challenges for marine nanotechnologists and ecotoxicologists lie in the identification of possible ENP transformations, interactions and behaviour in high electrolyte content aquatic compartments as well as elucidation of specific ENP modes of action towards living organisms (Schultz *et al.*, 2014).

Silver, long used as an industrial metal, has in recent times found widespread use in the form of nanoparticles (AgNP) for their antimicrobial properties. Indeed, silver nanoparticles,

with production volumes of 500 tonnes per year, are among the most used nanomaterials in consumer products - from cosmetics and dietary supplements to water purification systems, electronics and medical devices (Wijnhoven *et al.*, 2009). On the basis on L(E)C₅₀ values of ENP for environmentally relevant organisms, AgNP are classified as extremely toxic (L(E)C₅₀ < 0.1 mg L⁻¹), and generally show the highest toxic effects towards aquatic organisms (Bondarenko *et al.*, 2013; Kahru *et al.*, 2010). AgNP can remain relatively stable as they pass through freshwater systems (lakes, rivers) due to being complexed with dissolved organic matter and may eventually reach estuarine or marine environments as a final sink. However, more direct pathways for nanoparticle input to coastal areas are also possible, and may include offshore sewage outfalls, coastal septic tanks, direct input to estuarine systems and atmospheric deposition from coastal urban centres. In these compartments understanding AgNP behaviour and fate has been shown key to predicting their effects on biota and ecotoxicological potential (Chinnapongse *et al.*, 2011). Because of their specific physico-chemical properties, particularly in media of high ionic strength, they tend to agglomerate, aggregate and precipitate, with this behaviour modulated by dissolved organic matter such as alginate or humic substances, as well as AgNP original capping ligands such as, for example, citrate or protein (Angel *et al.*, 2013; António *et al.*, 2015, this issue; Dobias and Bernier-Latmani, 2013). These properties not only control agglomeration behaviour but may also play a role in the kinetics of silver ion (Ag⁺) release just as increased temperature and AgNP dilution promote release and higher pH and humic/fulvic acid reduce it (Hadioui *et al.*, 2013; Liu and Hurt, 2010). Furthermore, the bioavailability, uptake and accumulation of ENP play an important role on their toxicological potential by trophic transfer up the food chain (Behra *et al.*, 2013; Croteau *et al.*, 2014, Ward and Kach, 2009).

Although the rate and mechanisms of toxicity of AgNP is still not fully understood, Ag⁺ ion release plays an important role in the process (Faberga *et al.*, 2011). While acute Ag⁺ toxicity in marine invertebrates may involve inhibition of Na⁺, K⁺-ATPase in gill epithelial membrane and impairments in water and ion regulation at multi-organ cellular level (Bianchini *et al.*, 2005) the toxic effects derived from the specific properties of AgNP and ability to enter the cell and/or by generating the Ag⁺ ions and their pro-oxidation and pro-inflammatory activity has been established (Lapresta-Fernández *et al.*, 2011; Shultz *et al.*, 2014). Thus, several previous studies refer to this dual mode of AgNP action in aquatic organisms (Allen *et al.*, 2010; Chae *et al.*, 2009; Griffith *et al.*, 2009).

While much of the research on AgNP has focused on freshwater systems and organisms, there have been several articles focused towards the effects of AgNP on marine invertebrates (Bianchini *et al.*, 2007; Ringwood *et al.*, 2010; Gomes *et al.*, 2014). The sea urchin, as a globally distributed species, is widely used in embryology, developmental biology research and as a bioindicator. The ability for simple and routine *in vivo* laboratory cultivation throughout the larval stages of development makes them widely used as experimental models and markers of functional disorder. Recently, sea urchin embryos have been shown to be a suitable model for investigating the embryonal toxicity of different metal oxide nanoparticle species (Fairbairn *et al.*, 2011; Falugi *et al.*, 2011; Manzo *et al.*, 2013). Furthermore, Gambardella and co-workers (2014) demonstrated the trophic transfer of metal oxide nanoparticles by feeding *Paracentrotus lividus* larvae with nanoparticle-loaded marine microalgae which induced skeletal degradation, altered rudiment growth and reduced larval viability.

We have previously presented the first application of the sea urchin embryo development test in evaluating the ability of AgNP at very low concentrations to retard or prevent normal development of *P. lividus* larvae (Burić *et al.*, 2012). Similar findings were obtained by the exposure of *P. lividus* 4 cell stage embryos to significantly higher concentrations of citrate-stabilized AgNP (Šiller *et al.*, 2013). In addition, spermiotoxicity tests on *P. lividus* sperm confirm the impact of AgNP on embryonal development and skeletal biomineralization (Gambardella *et al.*, 2013, 2015). However, the potential for embryos to experience different levels of sensitivity at various stages of their development has not been addressed to date. Therefore, in the present study we aim to provide basic data on the effects of very low concentrations (down to 1 $\mu\text{g L}^{-1}$) of AgNP on sea urchin embryonal development, determine and contrast the sensitivity of different early life stages of three Mediterranean sea urchin species *Arbacia lixula*, *P. lividus* and *Sphaerechinus granularis* and investigate the possible impact of time at which developing embryos first encounter AgNP.

2 Materials and methods

2.1 Chemicals

Silver nitrate and tri-sodium citrate were purchased from Sigma Aldrich (St. Louis, MO, USA) at the highest analytical grade and used as received. Nitric acid used was 67-69%

Ultrapure for trace analysis (CARLO ERBA Reagents S.r.l., Italy). Silver and rhodium ICP-MS standards, at a concentration of 1000 mg L⁻¹ in 2% nitric acid were purchased from Absolute Standards Inc. (Hamden, USA). Ultrapure water (18 MΩ) used throughout the experiments was supplied by a Millipore Advantage System (Merck Millipore, Darmstadt, Germany) while filtered sea water (FSW) was obtained by filtering natural sea water (northern Adriatic Sea; salinity 38.1±0.1, pH 8.1±0.1) through Whatman 0.2 μm pore membrane filters (GE Healthcare Life Sciences, Little Chalfont, UK).

2.2 Synthesis of AgNP

AgNP were prepared by the sodium citrate reduction method. Briefly, 21.2 mg AgNO₃ diluted in 120 mL ultrapure water (18 MΩ) was heated until it began to boil. 5 mL of a 1% w/v sodium citrate solution was then added and the solution was held at boiling until the colour became pale yellow, upon which it was cooled to room temperature. This solution (final concentration 1 mM AgNO₃) was used as a stock nanoparticle solution for subsequent experimental work.

2.3 Sea urchin embryo development test

Specimens of black sea urchin *Arbacia lixula*, stony sea urchin *Paracentrotus lividus* and purple sea urchin *Sphaerechinus granularis* were obtained from the coast close to Rovinj, Croatia (northern Adriatic Sea) and kept for several days in outdoor aquaria containing natural sea water in a flow through system. Gametes were collected and eggs were fertilised as previously described by Quiniou *et al.* (1999) with slight modifications. Briefly, urchin gender was determined by injecting 100 μL 0.5 M KCl through the peri-oral membrane, shaking the urchins and observing the colour of the small volume of gametes which were released. Then approximately 1 mL of a 0.5 M KCl solution was injected again through the peri-oral membrane of an urchin and the urchin was shaken upon which gametes were released. Gametes were pooled from several individuals. Eggs were collected in FSW while sperm were collected dry and held on ice at 0 °C until use. Maturity of gametes was checked by confirming spherical eggs and mobile sperm. A 50 μL aliquot of oocytes in FSW (approximately 1500 mL⁻¹) and 10 μL of an 8-fold dilution of sperm in FSW was added to 20 mL FSW in Petri dishes, and were held at 20 °C (*S. granularis* at 16 °C). The samples were gently stirred to encourage fertilisation and then left untouched for 30 min (1 h for *S. granularis*) without additional aeration. Fertilisation success was found to be above 92% in all

experiments. The fertilised eggs were then exposed at various times post-fertilisation to either AgNO₃ or AgNP by the addition of appropriate volumes of the respective stock solutions such that final silver concentrations were the range of 1-100 µg L⁻¹. Times of first exposure to AgNP were 30 min, 90 min, 6 h and 24 h post-fertilisation for *A. lixula* and *P. lividus* while the procedure was modified for *S. granularis* with dosing carried out at 1, 6, 24 and 48 h post-fertilisation due to their slower rate of development. For comparison, embryos were exposed to AgNO₃ 30 min post-fertilisation (1 h for *S. granularis*) while a series of control samples were maintained without exposure to silver. The embryos were held in a natural daylight/night cycle with periodic agitation of the Petri dishes. After 48 h (96 h for *S. granularis*) larvae had reached the pluteus stage and were fixed in 0.1% buffered paraformaldehyde (40% w/v, pH 7.0) and scored for developmental abnormalities. All experiments were conducted in at least triplicate, with 100 random larvae counted for each replicate using a stereo microscope at 45× magnification, and a Nikon Microphot-SA compound microscope with CCD Nikon-Hitachi Camera (100× magnification) for documentation.

2.4 Statistical analysis

Differences among the percentage of normal, retarded and undeveloped sea urchin larvae following exposure to AgNO₃ or AgNP were determined by analysis of variance (ANOVA) followed by the conservative Bonferroni *post hoc* test (Systat 10.2; Systat Software Inc., San Jose, CA, USA). The levels of significance were ^α*p* < 0.05, [‡]*p* < 0.01 and ^{*}*p* < 0.001.

2.5 Instrumental characterisation

2.5.1 Nanoparticle characterisation

Aliquots of as-prepared AgNP stock solution were added to 0.22 µm filtered seawater, to a final Ag concentration of 1 mg L⁻¹, and UV-visible absorption data were collected periodically over a 72 h period on a Shimadzu UV-1800 spectrophotometer with a double beam configuration. Spectra were recorded in the wavelength range 300-800 nm at a resolution of 1 nm, with samples held in quartz glass cuvettes with an optical path length of 10 mm. Data processing was carried out on UVProbe 2.3.1 (Shimadzu, Kyoto, Japan) and Origin 9.0 (OriginLab Corporation, Northampton, MA, USA) software. Particle size distributions were determined by dynamic light scattering (DLS) of 1 µg L⁻¹ AgNP in ultra

pure water and in filtered seawater at various times after initial mixing and data were collected on a Zetasizer Nano ZS (Malvern Instruments, UK) instrument equipped with a green laser (532 nm). Samples were held in 10 mm path-length polystyrol/polystyrene cuvettes and intensity of scattered light was detected at the angle of 173°. Individual samples were measured a minimum of 10 times. The hydrodynamic diameter (d_h) of AgNP or agglomerates was obtained as the value at peak maximum of the size number distribution function. Data processing was carried out on proprietary Zetasizer software 6.32 (Malvern Instruments, UK).

2.5.2 Silver characterisation in water samples

To determine the extent to which silver nanoparticles release silver ions, aliquots of AgNP stock solution were diluted in filtered seawater to a final concentration of 1 mg L⁻¹ and held for different times from 0-48h with occasional agitation. The solutions were subsequently filtered through 3 kDa MWCO filters (Merck Millipore) by centrifugation and total silver content (as Ag⁺ ions) in the filtrate was determined by inductively coupled plasma mass spectrometry (ICP-MS). Measurements were carried out on an Agilent ICP-MS 7700x (Agilent Technologies, Santa Clara, USA) equipped with platinum sampling and skimmer cones, MicroMist quartz nebuliser and a quartz Scott spray chamber. Argon was used as carrier gas and helium as a collision gas in an Octopole Reaction System (ORS). The ICP-MS was operated in full quantification mode. Rhodium in 1% nitric acid was added on-line as an internal standard (ISTD) via a t-tube mounted before the nebuliser pump. Monitored signals included masses 107 and 109 for Ag and 103 for Rh, with isotope 107 on collision cell mode being used for quantification. A total of 6 silver concentration standards (plus blank) were prepared in 2% nitric acid in the range 0.2-50 µg L⁻¹. Calibration curves were read twice during the run and a total of five procedural blanks were analysed during the run. The Ag⁺ containing seawater samples were diluted 5 times in 1% nitric acid prior to analysis.

3 Results

3.1 AgNP characterisation

The UV absorbance spectrum of the as-prepared stock solution showed a strong peak with a maximum at 435 nm and is assigned to the surface plasmon resonance (SPR) of silver nanoparticles. This absorbance wavelength is consistent with nanoparticles of about 60 nm in

diameter (Gicheva and Yordanov, 2013). To probe the behaviour of the nanoparticles when introduced into the seawater and embryo -containing Petri dishes an aliquot of the nanoparticle stock solution was placed in natural filtered seawater and the absorbance spectra were recorded over the same period of time as the embryo development tests. The peak absorbance was observed to both shift to lower wavelength and decrease over 72 h, with the greatest reduction occurring within the first 4 h, as a result of nanoparticle agglomeration caused by the high salt content (Fig. 1). However, the continued persistence of the SPR may be related to the presence of natural organic matter which stabilises the nanoparticles to some degree and slows the agglomeration process.

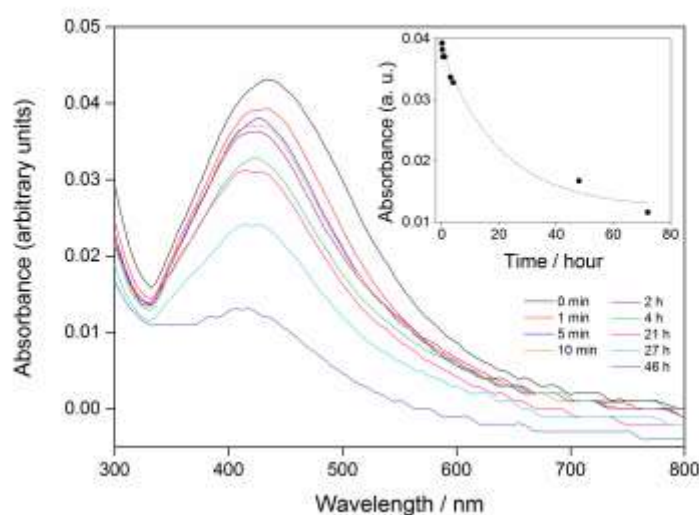


Figure 1 Change in absorbance spectra of AgNP in filtered sea water with time. Inset: exponential decrease of absorbance at peak maximum with time.

The hydrodynamic diameter of the as-prepared silver nanoparticles was determined by DLS and an average size of 59.67 ± 3.06 nm (10 measurements) was noted. The polydispersity index (PdI) of 0.42 ± 0.2 (Fig. 2.) suggests the presence of a range of nanoparticle sizes indicating that the synthesis has not produced nanoparticles with a strictly controlled size distribution. The behaviour of nanoparticles in filtered seawater was also investigated over the course of two days and two size populations were consistently observed in all samples (Fig. 2). These populations consisted of small and large agglomerates, typically in the range of 70-150 nm and 350-550 nm, respectively, based on number size distribution data. Considered in combination with UV absorbance data it is clear that individual or small agglomerates of nanoparticles remain present in the environment of the urchin embryos not

only in the period immediately after introduction of nanoparticles but throughout their development phase to the end of the experiment.

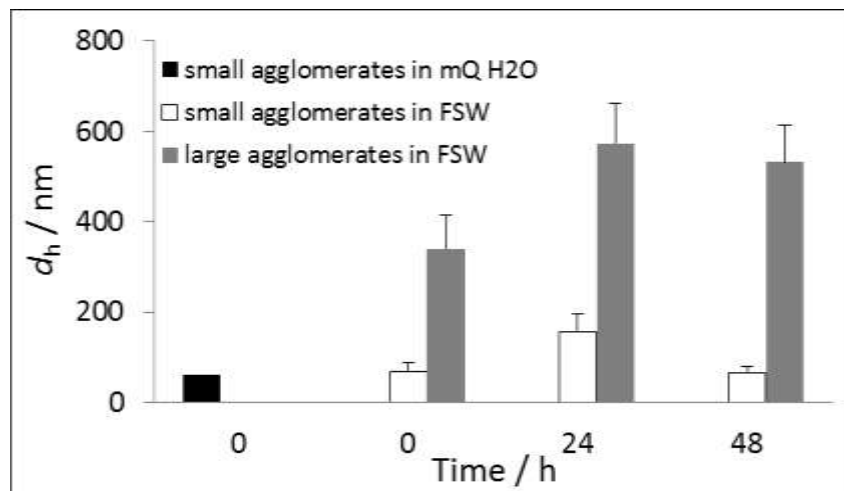


Figure 2 Hydrodynamic diameters (d_h) and standard deviations of freshly prepared AgNP in ultrapure water (mQ H₂O; black bar), and small (white bars) and large (grey bars) agglomerates in filtered sea water (FSW) after various periods of time (Ag concentrations were 1 $\mu\text{g L}^{-1}$).

To investigate the contribution of ionic silver to the total silver content in the Petri dishes during embryonal development, the concentration of Ag⁺ ions was determined by adding aliquots of AgNP stock solution to FSW (final concentration 2 mg L⁻¹) and subsequently filtering this solution using a 3 kDa cutoff filter (Amicon-Ultra centrifugal filter, Merck Millipore) after various time periods. The filter pores were sufficiently small to retain the nanoparticles while allowing Ag⁺ and related salts to pass through. The mass fraction of Ag⁺ with respect to the initial concentration of added AgNP solution was found to be relatively constant over a period of two days with values in the range of 2.7 - 6.8% given by ICP-MS analysis (Fig. 3). These data suggests that the majority of silver present is in the form of nanoparticles and no large-scale dissolution of these had occurred under the experimental conditions reported herein.

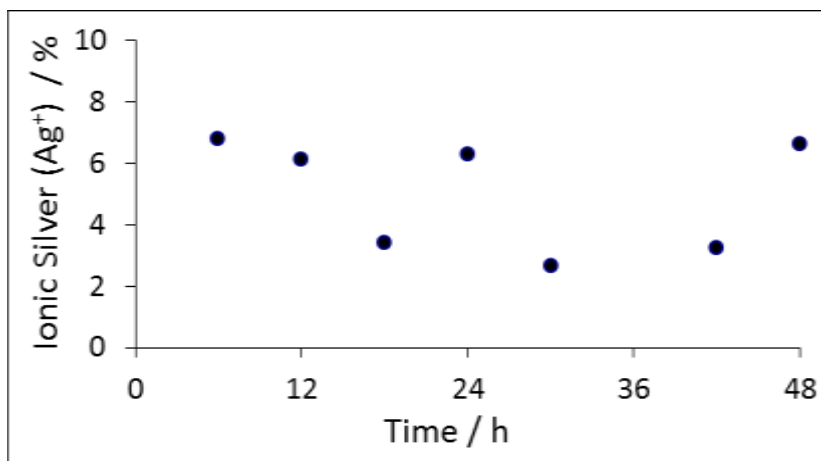


Figure 3 Silver ion mass fraction in filtered seawater with respect to initial mass of AgNP.

3.2 Morphological changes during Mediterranean Sea urchin embryonal development derived from AgNP

Developmental abnormalities of the embryos were classified in a manner which takes into consideration standard criteria used in the sea urchin embryo development test and, where possible, skeletal criteria as previously reported by Carballeira *et al.* (2012). Specifically, normally developed larvae were considered those that showed characteristic cone shape and morphology with developed arms and properly developed skeletal rods (Fig. 4 A-C) while retarded larvae were considered those at least two times smaller than the corresponding control larvae. These larvae also often showed specific deformations including crossed, separated or folded tip, fused arms, as well as incomplete or absent skeletal rods, thus deformed embryos were also included in the group encompassing ‘retarded’ development (Fig. 4 D-I). Undeveloped embryos were considered those of the prepluteus stage, absence of any skeletal elements or any embryos with arrested development (Fig. 4 J-L).

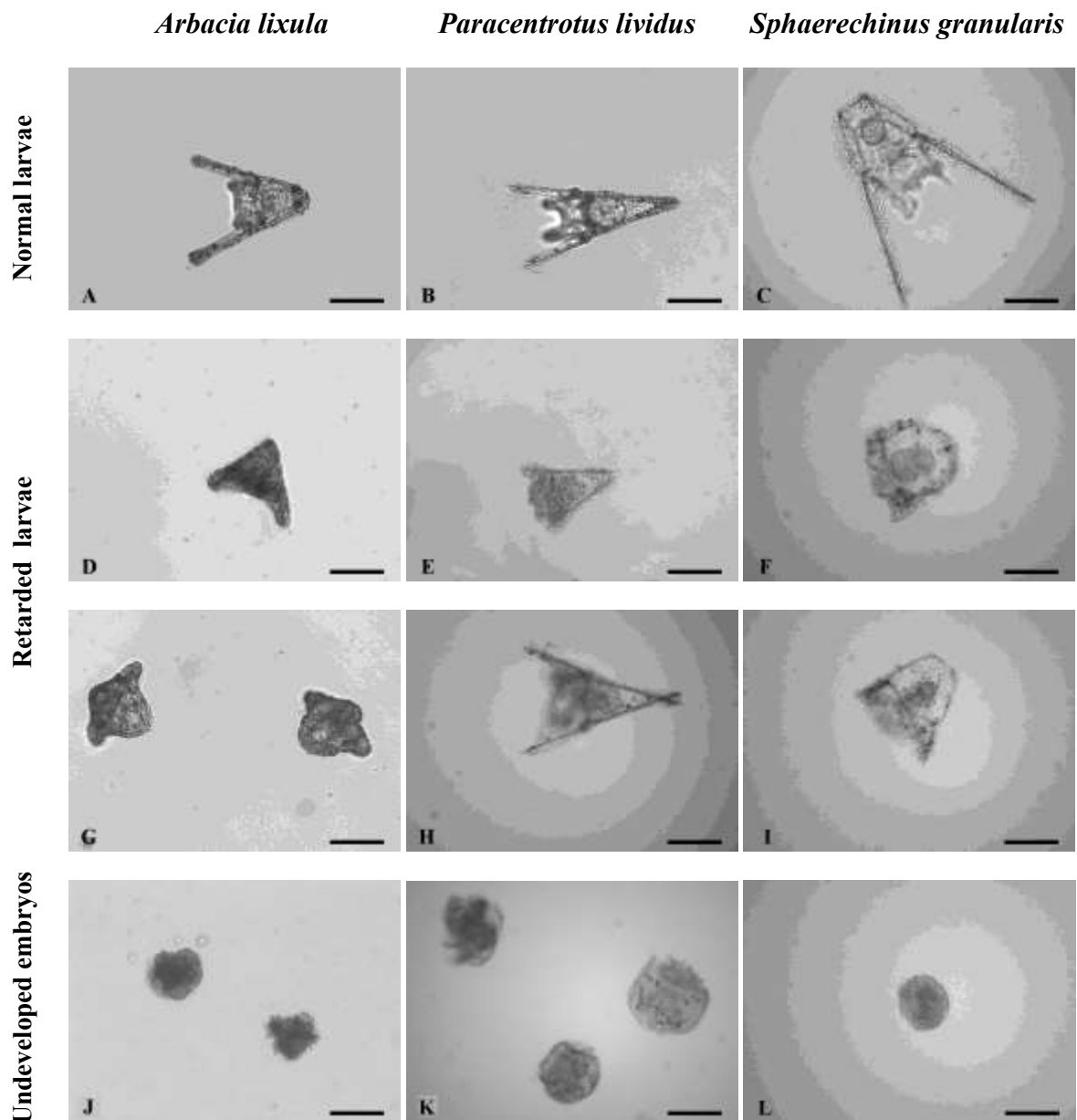
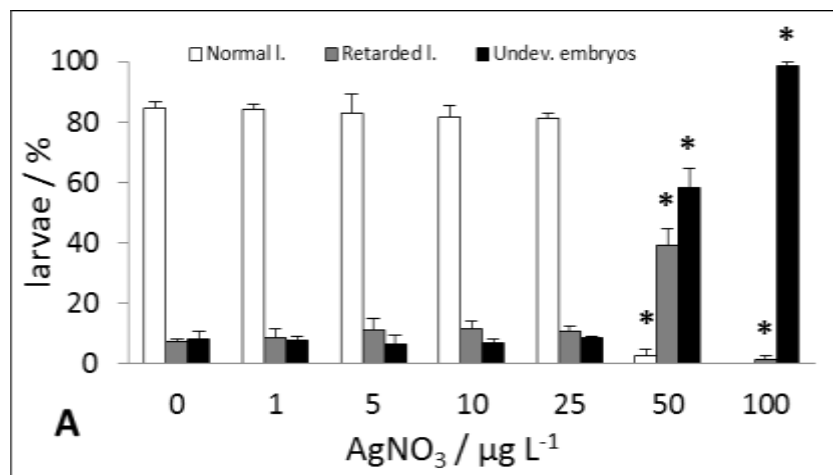


Figure 4 Normal (A-C), retarded (D-I) and undeveloped (J-L) *A. lixula* (column 1), *P. lividus* (column 2) and *S. granularis* (column 3) larvae after 48 h or 96 h development when exposed to AgNP (scale bar = 100 μ m).

3.3 The effect of $AgNO_3$ on development of Mediterranean Sea urchin embryos

Sea urchin embryos from the three species exposed to 1 - 100 μ g L⁻¹ Ag⁺ (as AgNO₃) 30 min post-fertilisation showed different effects on larval early stage development. *P. lividus* embryos treated with 1 to 25 μ g L⁻¹ Ag⁺ showed no difference in comparison to the control (non-treated) sample, but the higher concentrations of 50 and 100 μ g L⁻¹ caused significant changes in the number of retarded and undeveloped larvae after 48 h of development (Fig. 5A). 50 μ g L⁻¹ Ag⁺ exposure led to a significant decline of normal larvae from more than 80.0% to 2.5% and a simultaneous 4-fold increase (to 39.3%) of retarded and 7.5-fold increase (to

58.3%) of undeveloped larvae. The exposure to $100 \mu\text{g L}^{-1} \text{Ag}^+$ caused an almost absolute block (98.8%) of development. Similar results were obtained in experiments with *S. granularis* embryos with a noticeable difference at $50 \mu\text{g L}^{-1} \text{Ag}^+$ when the number of normal and retarded larvae were nearly equal (46.0% and 49.5% respectively) and significantly different both from the control and to larvae exposed to lower Ag^+ concentrations (Fig. 5C). The most sensitive embryos to Ag^+ at first exposure 30 min post-fertilisation appear to be those of *A. lixula* as even the very low Ag^+ concentration of $5 \mu\text{g L}^{-1}$ caused a significant difference in the ratio of retarded to normal larvae in comparison to the control sample. This trend is clearly visible up to $25 \mu\text{g L}^{-1} \text{Ag}^+$ exposure, while the highest concentrations led to a significant decrease of normally developed larvae to 6.5% ($50 \mu\text{g L}^{-1}$) and a significant increase to 92.0% and 97.0% (for 50 and $100 \mu\text{g L}^{-1} \text{Ag}^+$ concentrations, respectively) undeveloped embryos 48 h post-fertilisation (Fig 5B). Thus, although the highest Ag^+ concentrations tested here did not result in undeveloped or arrested development of *A. lixula* larvae, overall comparison of results for all three species indicated that sensitivity of sea urchin embryos to post-fertilisation exposure of Ag^+ decreases from *A. lixula* to *P. lividus* to *S. granularis*.



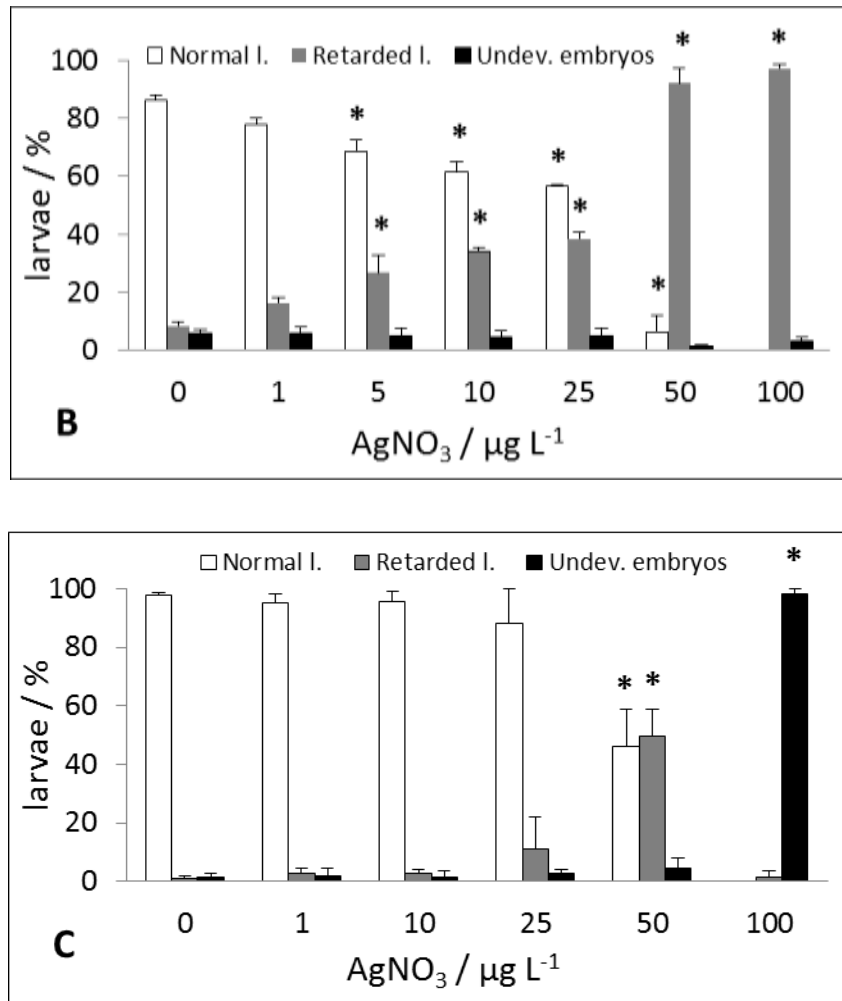
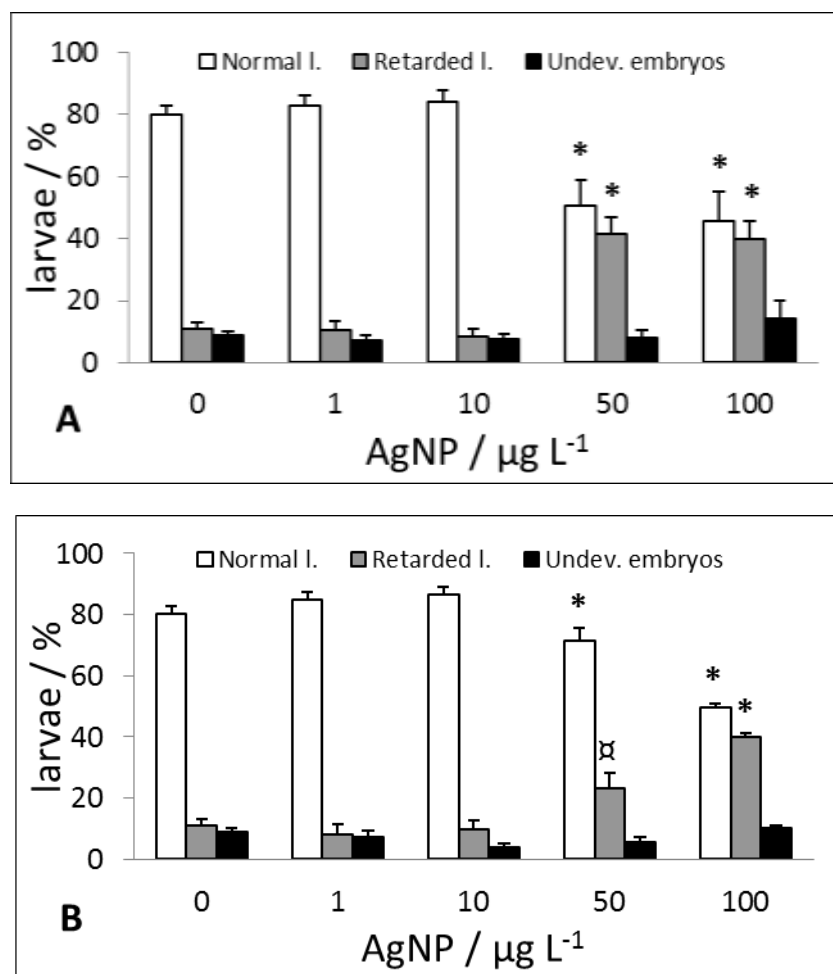


Figure 5 Percentage of normal (white bars), retarded (grey bars) and undeveloped (black bars) A) *P. lividus*, B) *A. lixula* and C) *S. granularis* larvae/embryos exposed to AgNO₃ 30 min (*S. granularis* 1 h) post-fertilisation. Significance levels denoted as **p* < 0.001.

3.4 The effect of AgNP on sea urchin embryo development

P. lividus embryos were exposed to 1 - 100 µg L⁻¹ AgNP at 30 min, 90 min, 6 h and 24 h post-fertilisation. For AgNP concentrations of up to 10 µg L⁻¹ the developmental patterns of the larvae remained relatively unchanged with respect to the control with the number of normally developed larvae greater than 80.0% (Fig. 6). However the dose of 50 µg L⁻¹ AgNP gave rise to significantly lower number of normally developed larvae, with a concomitant rise in number of retarded larvae exposed 30 min, 90 min and 6 h post-fertilisation (Fig. 6A, B, C). The lowest number of normally developed larvae (50.7%) and highest number of retarded plutei (41.3 %) were recorded in the 30 min post-fertilisation exposure experiment. Exposing the embryos to AgNP at successively later times after fertilisation resulted in greater success of the larvae reaching the pluteus stage with 71.0%, 66.0% and 74.0% fully developed after

first exposure at 90 min, 6 h and 24 h, respectively. Although embryos exposed to $100 \mu\text{g L}^{-1}$ at 30 and 90 min post-fertilisation showed no significant difference of undeveloped embryos to the control or to embryos which had been exposed to lower doses of AgNP, the number of normal and retarded plutei were significantly different to the control. Unusually, those embryos first exposed to $100 \mu\text{g L}^{-1}$ AgNP 6 h and 24 h post-fertilisation showed a significant increase of the undeveloped larvae fraction of 18.0% and 67.0%, respectively (Fig. 6C, D). This may suggest that *P. lividus* is less sensitive to AgNP shortly after fertilisation compared to later stages of development and may be related to the selective permeability and protective abilities of the fertilisation membrane with respect to very dilute solutions of AgNP.



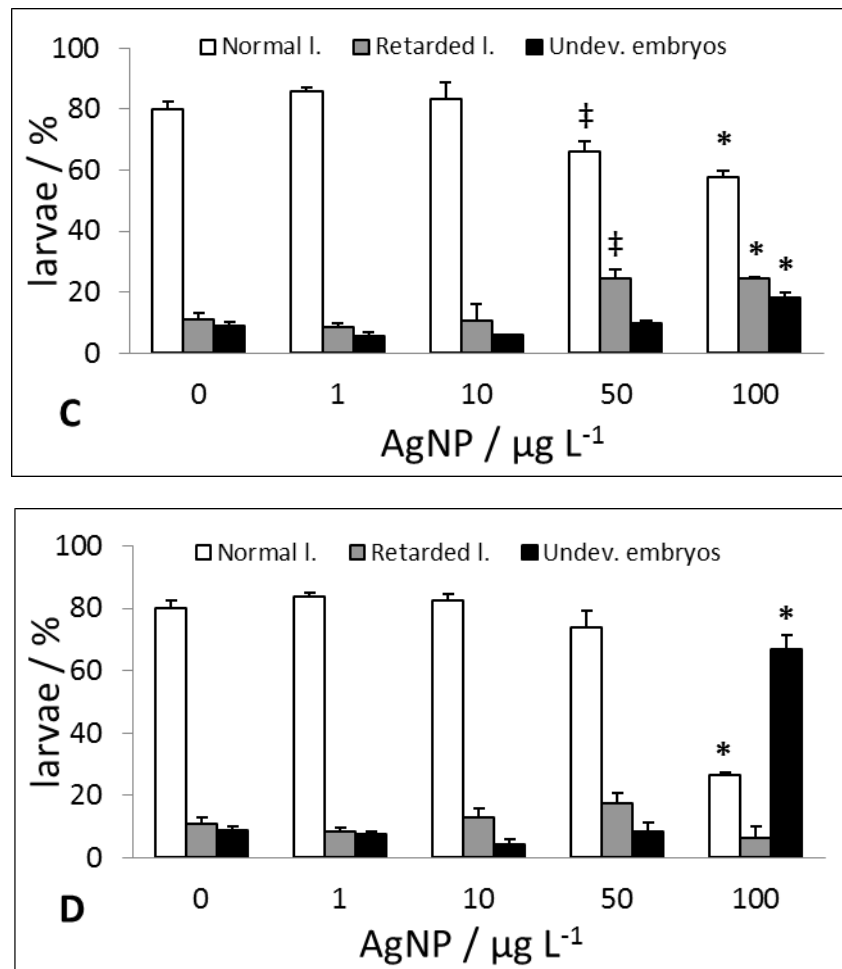
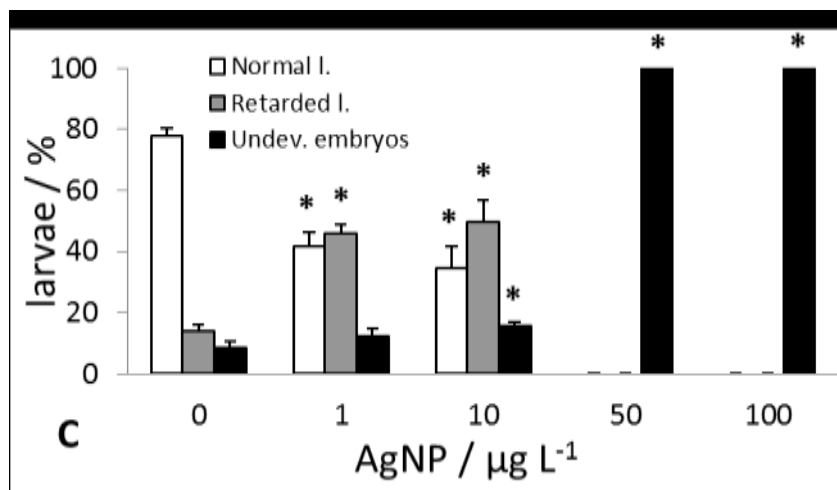
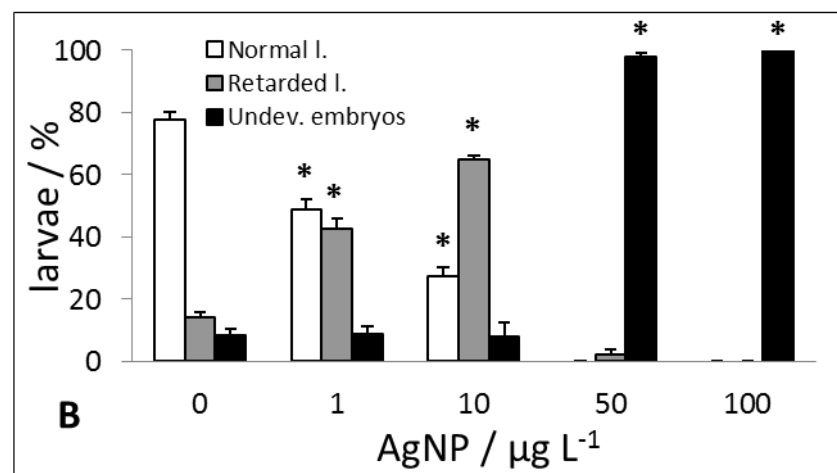
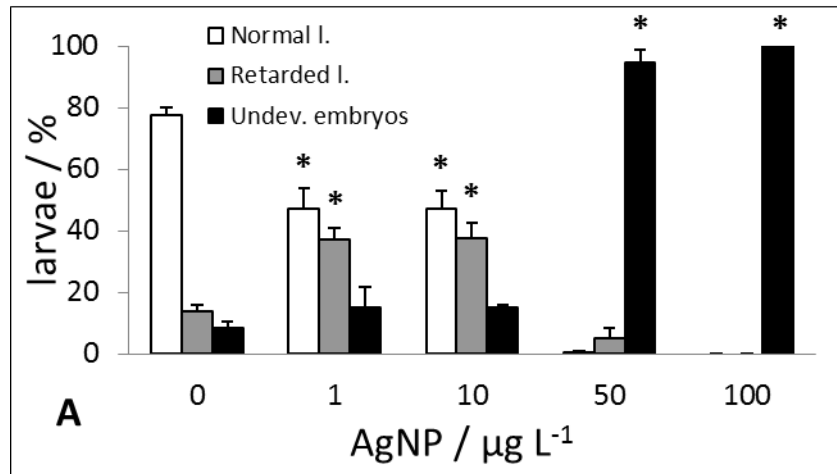


Figure 6 Percentage of normal (white bars), retarded (grey bars) and undeveloped (black bars) *P. lividus* larvae/embryos exposed to AgNP A) 30 min, B) 90 min, C) 6 h and D) 24 h post-fertilisation. Significance levels denoted as [†] $p < 0.05$, [‡] $p < 0.01$ and ^{*} $p < 0.001$.

A. lixula embryos were also exposed to AgNP in the concentration range of 1 - 100 $\mu\text{g L}^{-1}$ at 30 min, 90 min, 6 h and 24 h post-fertilisation (Fig. 7). The 30 min post-fertilisation exposure resulted in significant modulation of larval development in the entire range of applied doses. Specifically, the lowest concentrations of 1 $\mu\text{g L}^{-1}$ AgNP induced a significant 1.6-fold decrease of normally developed larvae (from 77.7% to 47.7%), and 3-fold increase of retarded larvae from 14.0% to 37.3 % (Fig. 7A). Further, the 6 h and 24 h post-fertilisation exposure to the lowest concentrations of 1 and 10 $\mu\text{g L}^{-1}$ AgNP induced large changes in the ratio between normally developed and retarded larvae such that the number of retarded larvae exceeded the number of normally developed larvae (Fig. 7C, D). The 90 min and 6 h post-fertilisation exposure to 10 $\mu\text{g L}^{-1}$ AgNP resulted in a similar outcome to the 30 min post-fertilisation exposure experiment with the slightly higher numbers of retarded larvae of 64.7% and 49.7%, respectively, being significantly different from the control. The 24 h post-

fertilisation exposure to 1 and 10 $\mu\text{g L}^{-1}$ AgNP resulted in a decrease in the number of normally developed plutei to 25.0% and 21.7%, while the fraction of retarded larvae was much greater, 60.3% and 61.7% respectively. The higher concentrations of 50 and 100 $\mu\text{g L}^{-1}$ AgNP resulted in a near complete block in embryonal development at all times of first exposure post-fertilisation.



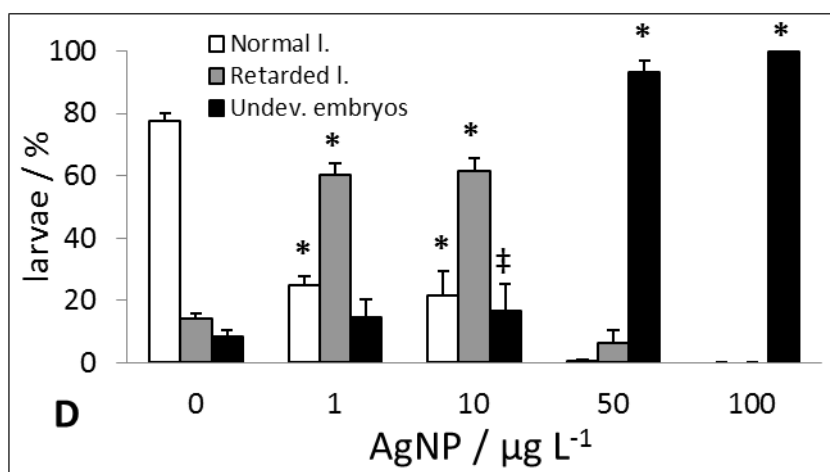
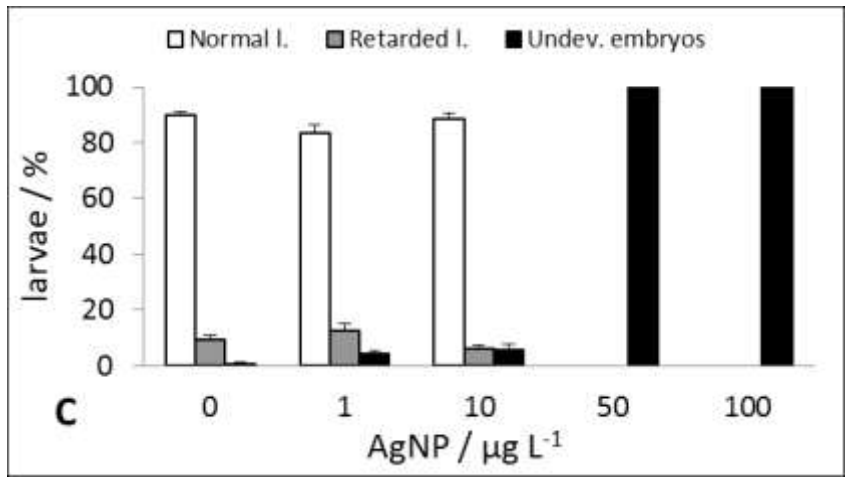
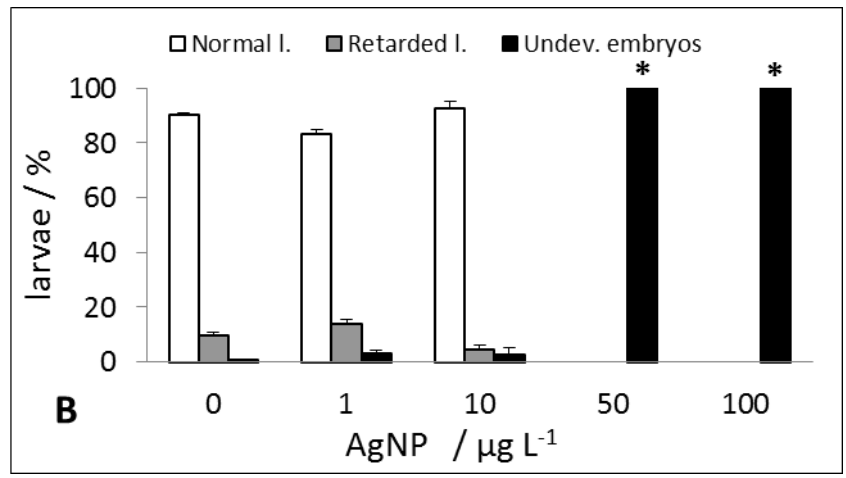
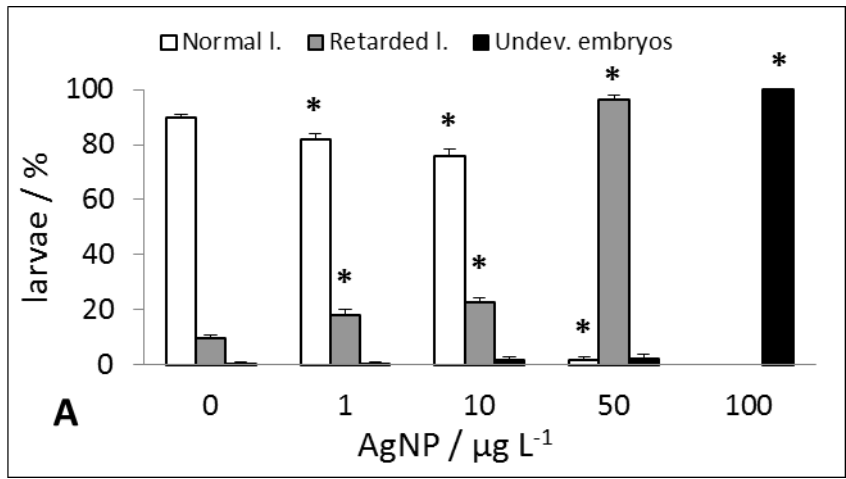


Figure 7 Percentage of normal (white bars), retarded (grey bars) and undeveloped (black bars) *A. lixula* larvae/embryos exposed to AgNP A) 30 min, B) 90 min, C) 6 h and D) 24 h post-fertilisation. Significance levels denoted as † $p < 0.01$ and * $p < 0.001$.

As with the other species, *S. granularis* embryos were also exposed to the same range of AgNP concentrations (1 - 100 $\mu\text{g L}^{-1}$) although the times of dosing were changed to 1 h, 6 h, 24 h and 48 h post-fertilisation. Similar to the 30 min post-fertilisation exposure of *A. lixula*, the 1 h post-fertilisation exposure of *S. granularis* embryos resulted in significant modulation of their development in the entire concentration range of applied doses (Fig. 8A). The number of normally developed larvae significantly decreased from 90.0% in the control sample to 81.7%, 75.8% and 1.5% in samples exposed to 1, 10 and 50 $\mu\text{g L}^{-1}$ AgNP, respectively. Simultaneously, the fraction of retarded larvae increased from 9.5% to 96.5%, while exposure to 100 $\mu\text{g L}^{-1}$ induced a complete development block. The 6 h and 24 h post-fertilisation exposure to 1 and 10 $\mu\text{g L}^{-1}$ AgNP resulted in relatively little disruption to the normal development of larvae, even though the differences were found to be significant with respect to the control. The doses of 50 and 100 $\mu\text{g L}^{-1}$ AgNP caused an absolute absence of normal or retarded larvae for first exposure at 6 h and 24 h post-fertilisation (Fig. 8B, C) while for earlier and later first exposure times of 1 h and 48 h, a concentration of 50 $\mu\text{g L}^{-1}$ AgNP resulted in nearly exclusively retarded development, and 100 $\mu\text{g L}^{-1}$ entirely blocking development (Fig 8A, D).



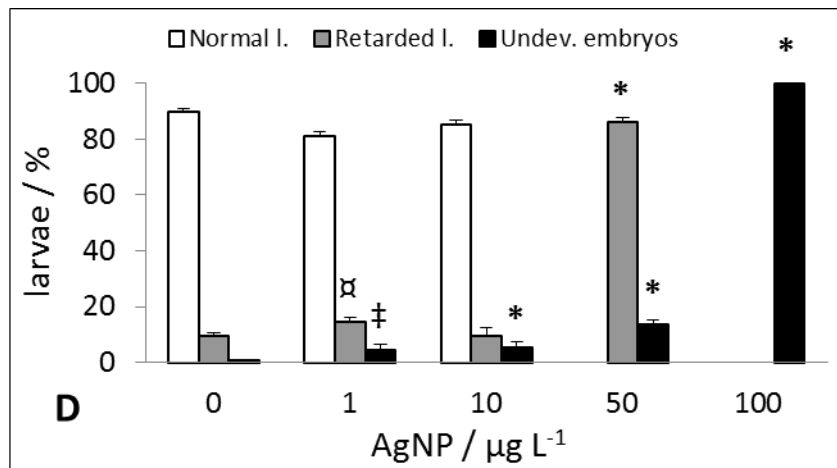


Figure 8 Percentage of normal (white bars), retarded (grey bars) and undeveloped (black bars) *S. granularis* larvae/embryos exposed to AgNP A) 1 h, B) 6 h, C) 24 h and D) 48 h post-fertilisation. Significance levels denoted as ^α $p < 0.05$, [†] $p < 0.01$ and ^{*} $p < 0.001$.

4 Discussion

It has already been shown that heavy metals, persistent organic pollutants and xenobiotics may affect several sea urchin species during early stages of development and induce different processes resulting in fertilisation failure, skeletal deformations, reduced gut elongation, delay in development or tolerance to temperature stress (Anselmo *et al.*, 2011, 2012; Kobayashi *et al.*, 2004; Quiniou *et al.*, 1999; Roccheri *et al.*, 2004).

The present study extends this area of research to engineered nanoparticles, gaining knowledge of the comparative effects of post-fertilisation exposure of *A. lixula*, *P. lividus* and *S. granularis* sea urchin embryos to AgNP. This research has specifically focused on how relatively low concentrations of AgNP affect the embryos of different species when those embryos are first exposed to AgNP at different times in their early development in terms of teratogenic effects such as arrested or retarded development or malformations in the embryo.

The experimental post-fertilisation exposure time points were chosen according to the different early life phases of the embryos as tracked in the laboratory under these specific experimental conditions. The first 30 min post-fertilisation exposure time was chosen as a sufficient time to allow fertilisation to take place, while the 90 min mark corresponded to 4 cell stage, 6 h to blastula and 24 h to gastrula stages (data not shown). As previously noted, those time-points were modified for *S. granularis* embryos, due to their slower rate of development at the lower temperatures preferred by that urchin, and were chosen at 1, 6, 24 and 48 h.

The results of the earliest exposure time point (30 min for *A. lixula* and *P. lividus*, and 1 h for *S. granularis*) to Ag^+ and AgNP suggested the existence of additional mechanisms of toxicity related to the action of AgNP, as greater teratogenic effects were noted for the AgNP samples than those containing an equivalent mass of Ag^+ . The toxicity of Ag^+ is well known (Bianchini *et al.*, 2005; Wijnhoven *et al.*, 2009) and while it should be noted that the AgNP released at least 6% of its mass as Ag^+ over the course of the experiment assuming low flux from dynamic metal speciation, the majority of silver likely remained in the form of nanoparticles as no strongly oxidising conditions were present during the experiment. Thus, while effects from AgNP-derived Ag^+ were not negligible, the overall enhanced toxicity of AgNP suggests a different mode-of-action related to nano-size, with effects that were ultimately found to be dose dependent and species specific.

This was further illustrated when the exposure of *A. lixula* embryos to 1 and 10 $\mu\text{g L}^{-1}$ AgNP and 25 $\mu\text{g L}^{-1}$ AgNO_3 induced similar effects (Fig. 5B, 7A). Furthermore, the highest dose of Ag^+ induced less deleterious developmental effects than the corresponding concentrations of AgNP. In contrast, *P. lividus* embryos exposed to low concentrations of silver, irrespective of the form, showed similar levels of toxicity while concentrations of 50 and 100 $\mu\text{g L}^{-1}$ Ag^+ showed higher levels of harm to embryos with respect to retardation or blocking of development compared to the corresponding AgNP-treated samples (Fig 5A, 6A). This clearly highlights how the effects of ionic silver and silver nanoparticles are species specific. *S. granularis* embryos proved to be sensitive to low concentrations of AgNP like those of *A. lixula* with a significant rise in number of retarded larvae by exposure to low doses of AgNP and absence of a similar effect when treated with the corresponding low doses of Ag^+ . At higher silver concentrations *S. granularis* mirrored the behaviour of *P. lividus* where the negative effects of 50 $\mu\text{g L}^{-1}$ Ag^+ on embryo development was greater than the effect of the corresponding AgNP exposure (Fig 5C, 8A).

Apart from increased mortality of urchin embryos and larvae due to AgNP, very low AgNP concentrations may also give rise to a range of sub-lethal effects such as, for example, immunosuppression (Falugi *et al.*, 2012). AgNP and/or Ag^+ may also cause disruption to normal physiological processes during early development and urchin growth and survival, including changes to motility and feeding patterns and difficulties in the ability to reproduce. As urchins occupy an important environmental niche, any such effects would be expected to have broader ecological implications.

The relatively low dissolution rate of AgNP under the experimental conditions kept the Ag⁺ mass fraction in FSW in the range of about 3 - 7 % with respect to initial nominal AgNP concentrations over a period of 48 h. Šiller *et al.* (2013) measured AgNP-derived ionic silver in seawater and found it to be about 1.0 % Ag⁺. Instead of comparing equal masses of ionic and nanoparticle silver as in the present work these authors took a different approach in that they compared the effects of 0.03, 0.3 and 3.0 mg L⁻¹ AgNP and 100-fold less Ag⁺ (0.3, 3.0 and 30.0 µg L⁻¹; corresponding to the amount of Ag⁺ derived from the nanoparticles). While using much higher nanoparticle concentrations than in our work they also concluded that AgNP have a higher negative impact on embryonal development and toxicity than Ag⁺. Thus their findings are broadly in line with our results for *A. lixula* and *S. granularis* embryos exposure to nitrate-derived Ag⁺ and AgNP 30 min post-fertilisation. Interestingly, their findings with respect to *P. lividus* varied from those found in this study indicating that other factors such as nanoparticle size (5-35 nm) or time of first exposure (2 h post-fertilisation in their case), and hence possible effect of fertilisation membrane permeability, play key roles the mechanism of which still need to be resolved. Further, these effects are certainly influenced by AgNP and Ag⁺ bioavailability in seawater (Faberga *et al.*, 2011; Behra *et al.*, 2013) with modes of toxicity deriving from 1) AgNP release of Ag⁺ ions upon which they may enter cells, 2) charge on the AgNP surface and subsequent disruption of cell membranes and 3) entry of the entire nanoparticle into the cell (Trojan-horse mechanism; Park *et al.*, 2010).

Similarly, Šiller and co-workers (2013) described a significant impact and induction of severe morphological changes with defects on the skeletal parts of developing embryos of *P. lividus* at very high AgNP concentrations of 300 µg L⁻¹ AgNP while no significant difference from the control was noted for 30 µg L⁻¹ AgNP exposure. In our opinion AgNP concentrations ≥50 µg L⁻¹ induce substantial malformations and retardation of *P. lividus* embryonal development regardless of the post-fertilisation exposure time-point up to 24 h and related development/presence of the plasma membrane. From the perspective of arrested development *P. lividus* embryos showed the greatest resistance to post-fertilisation exposure to AgNP where exposure to the highest concentrations up to 24 h post-fertilisation did not result in complete arrested development unlike for *A. lixula* and *S. granularis*. The reason for such behaviour may lie in slight but important modulations in metabolic pathways (e.g. possible chaperon or multi-xenobiotic resistance (MXR) activity) of the developing sea urchin embryos making some of them more resistant (*P. lividus*) and able to adapt to the

presence of AgNP. Unusually, exposure of *P. lividus* larvae to the highest concentration of AgNP at 24 h post-fertilisation induced significant developmental defects in more than 75% of the embryos (retarded + undeveloped; Fig 6D) despite the fact that the embryos had a shorter total exposure time to AgNP of 24 h (i.e. to the 48 h mark when the larvae were fixed and scored) than those of the other treatments. It is possible that the larvae had a gut developed at these later stages of the experiment potentially opening a new route to toxicity such as, for example, some internal uptake of nanoparticles as agglomerates.

However, caution must be used when comparing different exposure time points for a particular species as the developing embryos had different total exposure times to AgNP. Our two early exposure times of 30 and 90 min post-fertilisation have embryos/larvae under the influence of AgNP for nearly the same total time, i.e. 47.5 and 46.5 h for *P. lividus* and *A. lixula* and 95 and 90 h for *S. granularis*, so a qualitative or semi-quantitative comparison between these two exposure times may be possible in this case. Moreover, to allow more direct comparison of the effects of AgNP on different embryo/larva life stages of a particular species it would be necessary to carry out short pulsed exposures at different developmental stages to allow drawing of broader conclusions about stage specific sensitivities.

Contrary to the expectation of near complete agglomeration soon after introducing AgNP to the FSW, the persistence of the surface plasmon resonance of AgNP even after 3 days may indicate that some proportion of nanoparticles remain unagglomerated, likely due to stabilisation by complexation of dissolved organic matter to the nanoparticles surface (António *et al.*, 2015). Indeed DLS data confirm that single nanoparticles or very small agglomerates still remain in FSW (in addition to the expected large agglomerates) after several days despite the high electrolyte strength. However, it would be speculative to assign the greatest cause of toxicity to individual nanoparticles at this stage as larger agglomerates are also present and, because of their relative bulk, may cause toxicity by a different mechanism to single nanoparticles in this complex medium.

Thus overall, the results obtained herein on the exposure of *A. lixula* embryos to AgNP and silver nitrate-derived Ag⁺ ions suggests that this species shows the highest sensitivity among all of the chosen sea urchin species, independently of the post-fertilisation time of exposure. The other two species broadly appear to be more robust, with *P. lividus* embryos seeming to be less sensitive to low concentrations of AgNP but more sensitive to Ag⁺ exposure than *S. granularis* embryos. In the context of environmental significance *A. lixula*

may thus prove to be particularly valuable as a test species and in cases may be more appropriate than the more commonly used *P. lividus* as its high sensitivity is likely to give positive results before other species, and thus may be considered protective of other species.

5 Conclusions

The effect of AgNP on Mediterranean sea urchin embryonal development is species-specific and depends on AgNP concentration and bioavailability. Significant developmental abnormalities were found even in the range of 1 – 10 $\mu\text{g L}^{-1}$ AgNP in *A. lixula* embryos, while *P. lividus* and *S. granularis* embryos appear to be less sensitive although the latter also shows some negative effects from AgNP at concentrations as low as 1 $\mu\text{g L}^{-1}$. The order of sensitivity, from more to less sensitive, is therefore *A. lixula* > *S. granularis* > *P. lividus*. This suggests that *A. lixula* as the most sensitive organism may be an appropriate choice for nanoparticle toxicity testing where its low tolerance level may be considered protective with respect to other less sensitive species. However, more research must be carried out to confirm this. Further, as the moment at which embryos first encounter AgNP is shown to be an important factor in the development of abnormalities, future applications of the sea urchin embryo development test for nanoparticle toxicity testing should carefully address the specific phase of development of embryos when nanoparticles are first introduced as well as their bioavailability and physical/chemical properties in the immediate environment of the embryos.

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