Sinteza i utjecaj nanočestica srebra na sperme morskog ježinca Arbacia lixula

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University undergraduate study programme - Marine Sciences

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Synthesis of silver nanoparticles and their impact on the sperm of sea urchin *Arbacia lixula*

Bachelor thesis

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Bachelor thesis

JMBAG: , full-time student

Field of study: Marine Sciences

Subject: Nanotechnology in Marine Research

Mentors: Assoc. Prof. Daniel Mark Lyons
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Pula, September 2017

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Ovaj rad, izrađen u Laboratoriju za morsku nanotehnologiju i biotehnologiju Centra za istraživanje mora Instituta Ruđer Bošković u Rovinju pod vodstvom izv. prof. dr. sc. Daniela Marka Lyonsa, predan je na ocjenu Sveučilišnom preddiplomskom studiju Znanost o moru Sveučilišta Jurja Dobrile u Puli radi stjecanja zvanja prvostupnik (*Baccalaureus*) znanosti o moru.

Voditelj Sveučilišnog preddiplomskog studija Znanost o moru je za mentora završnog rada imenovao izv. prof. dr. sc. Daniela Marka Lyonsa i za komentoricu mag. ing. mol. biotechnol. Petru Burić.

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Datum i mjesto obrane završnog rada: Rujan 2017.; Institut Ruđer Bošković, Rovinj

Rad je rezultat samostalnog istraživačkog rada.

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1. Introduction

Nanoparticles are not a human invention but have always been present in the environment, particularly as ash, desert dust, metal oxides from weathering of rocks, aerosols, etc. (Baker et al., 2014). Indeed, even some plants are able to synthesize nanoparticles as they are capable of metal ion uptake in polluted environments followed by subsequent reduction to the zero-valent metal form. Despite organisms evolving in nanomaterial-rich environments it is not known how they will be affected by the presence of increasing amounts of engineered nanoparticles (ENP) produced by various nano-biotech industries and now widely used in various fields, including medicine, cosmetics, computer hardware manufacturing, agriculture, dietary supplements, etc. (Burić et al., 2015). Indeed, because of their already widespread application as catalysts, drug delivery devices and antimicrobials etc., by 2015 the fields in which they are used were foreseen to reach a value $1.5 trillion (Nel et al., 2006). Considering the rapid development of those fields it is safe to assume that number will only keep growing in the future.

As a consequence of their production and use becoming greater year after year it is inevitable that a certain quantity of ENP will find its way to soil and wastewater streams and eventually reach riverine, estuarine and marine ecosystems (Handy et al. 2008a; Klaine et al., 2008). Thus the interaction of ENP reaching the marine ecosystem with a diverse range of organisms is unavoidable. This scenario is especially pronounced near large population cities on estuaries and in coastal areas where significant amounts of wastewater, which are not necessarily treated to a significant degree, are discharged daily.

With the increasing realization that ENP are entering brackish and coastal marine waters in ever greater quantities and the potential for impacting on organisms living in those environmental compartments, research evaluating the behavior and effects of various ENP in these environmental compartments has recently become the focus of much attention, particularly with increasing evidence indicating the toxicity of ENP to various organisms. It has recently been shown that sea urchin *Arbacia lixula* embryos exposed to low, environmentally relevant concentrations of silver nanoparticles (AgNP) showed a substantial increase of retarded larvae and undeveloped embryos (Burić et al., 2015). Similar results have also been reported for other urchin species including *Sphaerechinus granularis and Paracentrotus lividus*,

clearly indicating that ApNP negative effects may be broadly species independent. Apart from developmental defects, ENP have also been shown to cause a wide range of other negative effects including, for example, DNA damage in *Acaryochloris marina* exposed to TiO_2 nanoparticles (Galloway et al., 2010) or carbon nanotube–induced cytotoxicity in *Dunaliella tertiolecta* (Wei et al., 2010).

However, while research addressing these issues is increasing, there are still many unanswered questions and we remain unable to predict the impact of nanoparticles in various ecosystems on key species. To address this in part, and bearing in mind the ability of AgNP to impact upon embryonal development, the present study focuses on determining the effect of AgNP on the sperm of sea urchin species *Arbacia lixula* in terms of modulating their ability to fertilize urchin eggs and how embryos subsequently develop.

1.1 Nanoparticles

During the 1970s and 80s the first studies on particles less than 100 nm in size began to appear. While they were initially known as „ultrafine particles“ (Hayashi et al., 1997), the term „nanoparticles” became more common during the ‘90s. Nanoparticles are defined as particles that have dimensions between 1-100 nm and, due to their novel properties (catalytic, antimicrobial, vector, conductive, UV blocking) due to size-based modulation of their electronic energy levels, have been used in various fields including medicine, electronics production, cosmetics, chemistry, etc. (Schmid, 2010). Specifically, this includes polymeric micelle nanoparticles that deliver drugs to tumors, cerium oxide nanoparticles that remove free oxygen radicals, ceramic silicon carbide nanoparticles that dispersed in magnesium produce a strong yet lightweight material, gold nanoparticles as catalysts to breakdown volatile organic pollutants, titanium dioxide or zinc oxide nanoparticles in sunscreens as protection from UV radiation, nanoscale silicon transistors used as on and off switches in processors for binary information storage. The Woodrow Wilson Database (2011) has listed 1,317 nanoparticle based consumer products on the market, of which 311 contain silver nanoparticles (AgNP; Gambardella et al., 2015). Many of these products will likely contribute to the overall environmental load of AgNP and predicted

environmental concentrations of AgNP are already estimated to be between 0.03 and 0.08 µg/ L in aquatic environments (Fabrega et al., 2011).

By virtue of their broad and growing usage (especially in the cosmetics and food industries) and lack of data on their transformative and bioaccumulation properties, and hence their potential to lead to harmful effects, in 2009 the European Union implemented a new directive regarding the use of ENP as Regulation (EC) No. 1223/2009, including the strict protocols to be implemented before ENP-containing cosmetics may be released on the market including the gathering of toxicological data and labeling requirements stating that the product contains particles smaller than 100 nm (nanoparticles) (EU, 2009).

1.1.1. Properties

Due to their small size nanoparticles have novel and interesting properties that have been observed and described. Primarily, ENP show properties that are intermediate between those of the corresponding bulk material (larger than one micrometer) and molecular structures. It is well-known that bulk material properties do not change depending to size or amount of material (e.g. a cinder block of 10 cm^3 will not change color if the scale is increased to 1 m^3, or a small plaquette of 24 karat gold will be as shiny as a bigger 24 karat gold piece). However as the material get smaller the ratio between surface and volume becomes bigger, the electronic band structure becomes modified and as a result its properties change dramatically. Moreover, as the material gets smaller the wave aspect of wave-particle duality become more apparent.

Correspondingly, nanoscale materials behave differently from their bigger macroscopic counterparts at the fundamental property level. As stated by Hewakuruppu, Y. L. et al. (2015) This is particularly notable on the opto-electronic level where nanoparticles often produce unexpected optical properties since their valence or surface electrons are confined to different energy levels due to quantum effects resulting in nanoparticles interacting differently to light. Both size and shape influence electronic levels within nanomaterials. Silver nanoparticles' colour varies from yellow (Ag spheres of 40 nm) to pale yellow/grey (Ag spheres 80 nm) to red (Ag

nanoprisms 100 nm) (Horikoshi and Serpone, 2013). A further example is how the size of gold particles influences its colour, namely bulk gold appears yellow while 12 nm gold nanoparticles have a distinct red colour. Further, gold nanoparticles melt at significantly lower temperatures than bulk gold, for example, at 300°C for 2.5 nm gold nanoparticles, while bulk gold melts at 1064 °C (Buffat and Borel, 1976).

Nanoparticles of zinc oxide and titanium dioxide recently found extensive use in sunscreen because of their UV absorbance properties (TiO_2 at around 300 nm (UV-B) and ZnO at around 350 nm (UV-A) (Manaia et al., 2013). However, at the nanoscale, size also has a dramatic effect on the scattering of visible light. Peak scattering occurs when the particle size is equivalent to half of the visible light wavelength, assuming that the particles are uniformly dispersed as stated in Mie theory. In sunscreens, increased scattering occurs when the nanoparticles included in the formulation have sizes in the range of 200-300 nm give the so-called whitening effect. Thus sunscreen formulations tend to use TiO_2 and ZnO nanoparticles in the 20-50 nm range.

As previously mentioned, the high surface to volume ratio results in enhanced catalytic properties whereby the rate of reactions may be increased by orders of magnitude. This includes, for example, cobalt nanoparticle assisted oxidation of cyclohexane into adiapic acid (precursor of nylon) or cobalt nanoparticle assisted hydrogenation of aromatic amines for the synthesis of pesticides and herbicides (Roucoux et. al., 2002).

1.1.2. Characterization methods

There are various characterization methods that have been used for determining nanoparticle size, structure, composition, surface chemistry, etc. in recent years. However, every method presents advantages and disadvantages, with none of them able to present an unequivocal characterization of a sample, hence requiring that more than one method be used when describing the physico-chemical properties of ENP. In this Secion the main characterization methods used in nanotechnology will be briefly discussed, while the interested reader is directed to more comprehensive texts for a more detailed discussion of the following methods (e.g. Tantra, 2016).

One of the most important of these techniques is dynamic light scattering (DLS). It is used to determine the size of small particles (0.002 to 2 microns), in a very diluted suspension or solution, experiencing Brownian motion. The sample is illuminated with a monochromatic light (usually laser) that has passed through a polarizer, and as a consequence of the small particles the light is scattered at various intensities. Those fluctuations in intensity are detected by a photosensitive detector and using the Stokes-Einstein relationship the particle radius can be deduced. The main advantages include: the evaluation is fast (from seconds to minutes), any suspension or solution that isn't too viscous can be analyzed, and broad size range can be assayed. The main disadvantages include: interference due to dust, particle shape can only be obtained with some difficulty, and high resolution histogram of size distribution is not available.

A second technique is scanning electron microscopy (SEM). The sample is illuminated by a beam of electrons that interacts with the atoms of the sample. The sample's properties like composition, topography, etc. are determined by the detection of the signal in the form of scattered and secondary electrons and x-rays that have been generated. While images are of high resolution (better than 1 nm) and the 3D topography of the sample can be seen, this method is very time consuming and expensive. Furthermore, conductive surfaces are required for the very highest resolution work and internal structures in materials cannot be seen.

An extremely important characterization technique is transmission electron microscopy (TEM) that can be used to provide 2-D imaging to give sample size, shape and crystallographic structure. For the latter, the beam of electrons interacts with the sample by diffraction. The diffraction intensity depends on the orientation of the planes of atoms. The deflected electrons are recorded by detectors and the crystal structure is determined. Use of TEM is however a time consuming process and the instrument is expensive. Furthermore, the sample can't be frail and must be able to tolerate high vacuum and the impact of an electron beam. Moreover, the scanning footprint is very small, therefore the TEM technique may only show a few nanoparticles per unit area, hence this technique doesn't give data about the ensemble but rather limited snapshots of the sample. High resolution transmission electron microscopy (HRTEM) is also available, making imaging at the atomic scale possible.

Atomic force microscopy (AFM) as a technique can be used to display vertical resolutions of less than 0.1 nm and horizontal resolutions of 1 nm and is based on a minute needle that is brought near, or into contact with, the sample and moved along its surface. As the needle is moved across the surface a laser tracks its movement (that follows the sample's topography). The laser return time-signal deviation is used to convert the sample's profile into a topographic map of the surface. This approach in atomic force microscopy is called contact mode although, while allowing the best resolution, the sample can be damaged by the needle tip. However, modern atomic force microscopes have two other modes: non-contact mode and tapping mode. In the first the tip hovers above the surface, as a consequence attraction forces are measured. This method is used for frail samples, hence its non-destructive nature. However the image resolution is poor. In tapping mode, as the name suggests, the tip is repeatedly tapped on the samples surface. This mode provides a balance between sample resolution and damage incurred by the sample. Physical properties of a single particle such as morphology or surface texture can be measured, as well as statistics regarding groups of particles. Atomic force microscopy can be performed in all media (solid, liquid or gas) as well in both ambient air and controlled surroundings (such as vacuum, nitrogen or argon gas). Furthermore, particles from 1 nm to 5 μm in height can be measured in a single scan.

A related technique is scanning tunneling microscopy (STM). This method is based on quantum tunneling. When a conductive tip is brought close (less than 10 nm) to another conductive or semi-conductive surface there is a chance that electrons will "tunnel" or "jump" from the needle to the sample and vice versa, since their probability function may overlap. The fluctuation of tunneling electrons as the probe passes over the samples surface is rendered as an image. STM can show depth resolution up to 0.01 nm and lateral resolution of 0.1 nm. As with AFM, it can be used various media like: vacuum, air, liquid, specific gas, etc. and over a wide range of temperatures. However, extremely clean surfaces and ultra-sharp tips are required.

1.1.3. Fate and behavior in aquatic environments

With entry of ENP into the environment, there are likely to quickly enter freshwater systems. In these systems, nanoparticle fate and behavior is strongly linked to their proprieties: size, shape, surface charge, functionalization and coating (Canesi and Corsi, 2016). Further, these physical and chemical characteristics have a strong impact on their interactions with living organisms and hence potential toxicity. From freshwater systems, ENP may be transported to brackish and coastal waters although more direct modes of entry are also possible including atmospheric deposition, rainwater run-off and wastewater streams (Figure 1; Baker et al., 2013)

When ENP arrive in high salinity media various transformations can occur. A range of reactions can occur at the air-water interface (plankton absorption, aerosolization etc.). Secondly, they can be accumulated by different organisms causing accumulation and/or toxic effects. For example, the bivalve *Mytilus galloprovincialis* showed titanium accumulation in grills when exposed to titanium dioxide (TiO_2; Della Torre et al., 2015).

Further, they can dissolve and/or be transported through the water column making them bioavailable to pelagic organisms (through breathing, feeding, adsorption). An important aspect of ENP in terms of bioavailability are their agglomeration (weak forces like van der Waals bonding substances into agglomerates) or aggregation (strong chemical forces binding substances onto aggregates) characteristics (Tso et al., 2010). Aggregation and agglomeration are driven by 2 main factors. The first are the properties mentioned previously (size, surface charge etc.) while the second driver comprises several parameters of the media the nanoparticles are in, including pH, osmolarity (ionic strength), salinity and presence of natural organic matter (colloidal polymers yielded by algae known as exopolymeric substances or EPS showed notable capability of influencing aggregation; Canesi and Corsi, 2016).

As individual ENP, agglomerates or aggregates may become encapsulated with organic matter and detritus, and may slowly sink to the sea bottom where they encounter further changes in temperature, organic matter and dissolved oxygen. Upon reaching the ocean floor they may be absorbed by benthic organism and subsequently promoting toxic effects in those organisms. Secondly, sedimentation or

formation of a biofilm may occur, while subsequently, due to benthic organism activity, bio-turbation and re-suspension may make the nanoparticles again available to pelagic organisms.

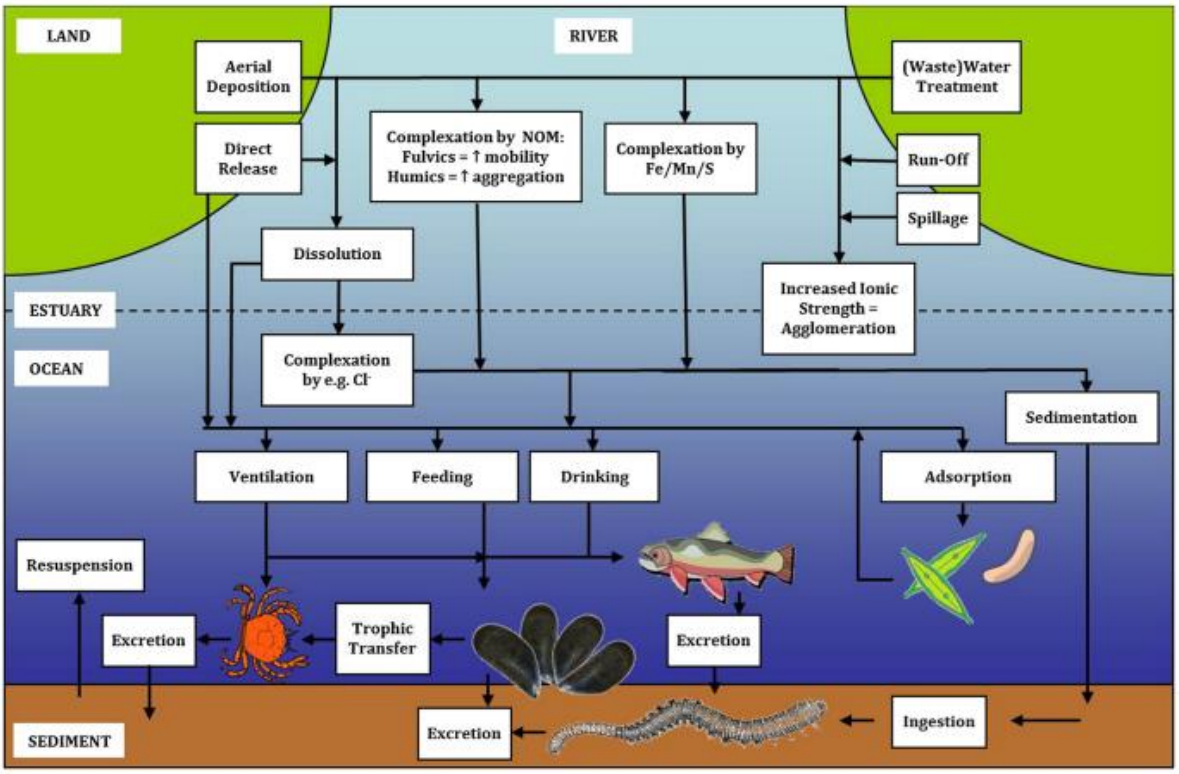

Figure1. Fate and behavior of nanoparticles in aquatic environments (*source:* Baker et al., 2013).

A further key process in the movement of ENP through the aqueous environment is their accumulation in one organism which is then eaten by another, thus providing a pathway into the food web and eventually biomagnifications up the food chain (Holbrook et al., 2008). This trophic transfer of ENP may eventually have consequences for human health should those organisms that may accumulate ENP be used as a food source, including bivalves and fish.

1.1.4. Uptake and bioaccumulation by aquatic animals

Recently several studies have been conducted, showing ENP are capable of producing toxic effects, lethal and sub-lethal, including oxidative stress, development retardation, reduced fertilization rate, etc. Freshwater species including *Daphnia magna*, *Lymnaea stagnalis* and *Caenorhabditis elegans* treated with ENP showed a variety of sub-lethal effects such as digestive stress and reduced feeding (Croteau et al., 2011), bioaccumulation (Rosenkranz et al., 2009) and reduced swimming (Asghari et al., 2012). More recently, studies have focused on sea urchins with detailed investigations of the impact of engineered silver nanoparticles on embryonal development and negative impacts were noted for very low ENP concentrations (Burić et al., 2015).

AgNP and silver ions (Ag^+) were also used to treat 2 different species: *Scrobicularia plana* (bivalve) and *Hediste diversicolor* (polychaeta; Buffet et. al., 2013). After 20 days of exposure both species showed statistically significant higher amounts of silver nanoparticles and silver ions in the organisms, thus indicating signs of bioaccumulation. Furthermore, as markers of oxidative stress, significantly higher glutathione S-transferase (GST), catalase (CAT) and expression of CSP (Central nervous system-specific proteins) were detected in both species (*S. plana* and *H. diversicolor*) exposed to both silver forms (nanoparticles and ions). In addition, higher SOD (superoxide dismutase) and TBARS (thiobarbituric acid reactive substances, as byproducts of lipid peroxidation) levels in *H. diversicolor* were observed, hence further indication of oxidative stress. In other similar work, the bivalve *Mytilus galloprovincialis* showed titanium accumulation in gills when exposed to titanium dioxide (TiO_2) nanoparticles (Della Torre et al., 2015).

Apart from filter feeders, studies have also shown that bacterial biofilms may significantly accumulate ENP (uptake of 60%). However *I. obsolete* had surprisingly low uptake (0.05%) despite its biofilm grazing habit (Ferry et. al., 2009). *Cyprinodon variegates*, the sheepshead minnow, has also been found to have a low uptake (Baker, T.J. et al., 2013). In similar studies, trophic transfer of metal oxide nanoparticles has been demonstrated by Gambardella et al. (2014). *Paracentrotus lividus* showed skeletal degradation and smaller larval growth when fed with nanoparticle loaded marine microalgae. Therefore it may be concluded that trophic

transfer and uptake of nanoparticles in the marine environment are complex mechanisms, and while possible, depends closely on uptake efficiency which is variable from organism to organism, hence more in-depth research is highly needed.

1.2 Sea urchins in scientific research

Sea urchins have found widespread use in marine research for their well-known biology, physiology and anatomy. In particular, urchins have been used in studies of embryonal development and immunology as aspects of their immune system functioning is analogous to the that of the human system in certain ways.

Sea urchins belong to the phylum Echinodermata (including sea stars, crinoids, etc.) and class Echinoeidea, with the first fossils date back to the Ordovician era. The class name when translated into english means “like a hedgehog” because of their spines enveloping their bodies. Their habitat spectrum is vast, ranging from the littoral zone (1 m depth) to abyssal depths of 5000 m. Some species prefer rocky depressions, while others are able to live burrowing themselves into soft sediment (irregular sea urchins). In general, urchins are small, typically from 3 to 10 cm in diameter, yet some species found in the Indo-Pacific area can reach nearly 36 cm in diameter. Their color varies, with black, purple, blue and red sea urchins can be commonly found, however, white and multicolored sea urchins may also be found occasionally. Sea urchins are generally round or oval animals, and in the latter case can be lightly or greatly flattened along the oral and aboral axis. Their bodies can be divided into two main sections, the oral and the aboral pole.

The oral pole is directed against the substrate and the oral cavity is covered by a peristomial membrane. Other structures that can be found in this region include the buccal podia (5 short modified podia) and the gills. The aboral region, also known as periproct, is a tiny and circular membrane containing a fluctuating amount of embedded plates (number varies from species to species) and the anal cavity, commonly found in the central region of the periproct.

A distinctive sea urchin feature is the exoskeletal structure, made by flattened ossicles that have been sutured together. The skeletal plates form rows running from the oral pole to the aboral region. The exoskeletal structure has protruding spines

arranged more or less symmetrically that are used for protection, movement and raising the oral cavity off the sediment. The spines are several centimeters long, with the longest being found in the equatorial area and shorter ones at the poles. Secondary (shorter) spines can be found in the equatorial area in some urchins such as *P. lividus*. The spines are hollow, cylindrical, regenerable and frail, with a sharp point at the end. They are coated with an irritant and small barbs pointing towards the sharp end, therefore painful wounds can be inflicted if stepped on with enough force. Blunt spines can be found in some species (genus *Colobocentrotus*), moreover some species of the *Echinothuridae* family have poison coated spines located in the aboral region.

Sea urchins, like other echinoderms in the early larvae stage, have bilateral symmetry, however with development their symmetry becomes radial and five-fold (Giudice, 1973). The spines and the podia are used in movement on hard and soft bottoms, in any direction. Movement is closely linked to feeding activity (in case of food abundance only 8 cm per day, although in oligotrophic areas there may be movement of up to 50 cm per day). Sea urchins have a well-developed scraping structure called Aristotle´s lantern that is used for feeding purposes. This organ is made of five calcareous plates called pyramids, arranged radially and pointing towards the oral cavity. The sharply ended pyramids are connected with transverse muscle fibers. Because of their grazing habits new tooth material is produced at a rate of 1.5 mm per week. Sea urchins are predominantly grazers, scraping algae with the highly developed Aristotle˙s lantern. Their main diet consists of algae, however animal material can be consumed. Conversely, urchins also represent prey, with starfish, eels and sea otters as their main predators. Coelomic fluid is the primary circulatory medium (gas exchange, internal transport and excretion) and sea urchins have a radial nervous system. Fertilization takes place in seawater after sperm and egg cells are released from 5 gonads directly into the sea. Brooding has been found in some sea urchin species, where the eggs are retained on the peristome or periproct and the spines are used to secure the fertilized eggs. Recently, sea urchins have been used as non-model organisms in a number of studies aimed at determing nanoparticle toxicity.

Urchins present a number of advantages for use in marine research; they are very common organism in the littoral area and their sampling is trivial due to their

small size and sedentary lifestyle; their habitat is important because the highest concentrations of nanoparticles will likely be found in coastal waters due to various rainwater run-offs, surface freshwaters and wastewater streams. Further, they may be useful sentinels due to their grazing habits as bioaccumulation may occur if toxins or ENP are found in algae. Moreover their embryo and larvae stages are fairly short and simply counted via optical microscopy, and their fertilization is easily replicated in captivity.

2. Research scope

Taking into consideration recent literature reports showing AgNP toxicity to both freshwater organisms and marine invertebrates including shellfish *Mytilus sp*. and sea urchins *A. lixula*, *P. lividus* and *S. granularis* as model organisms (Burić et al., 2012; Gomes et al., 2014; Burić et al., 2015) the present study focuses on extending these data to determining the impact of AgNP on sea urchin gametes.

As shown by Burić et al. (2012) even low concentrations of silver nanoparticles are able to retard or stop regular *P. lividus* larvae development. Further, in that study, different sea urchin species showed different sensitivity to AgNP at various stages of their larval development. However AgNP treatment response by gametes such as *A. lixula* sperm have not been researched in detail to date. Therefore, the objective of this study was to synthesize AgNP of a known size, and subsequently treat *A lixula* sperm with various concentrations of these AgNP. Afterwards the fertilization rates would be measured and the percentages of normal, retarded, undeveloped and dead larvae would be determined after allowing embryonal development to proceed for a defined time period.

3. Materials and methods

3.1 Chemicals

Silver nitrate (AgNO3), tri-sodium citrate ($C_6H_5O_7Na_3$), potassium chloride (KCl) of analytical grade purity were purchased from Sigma Aldrich (St. Louis, MO, USA). A Millipore Advantage System (Merck Millipore, Darmstadt, Germany) supplied ultrapure water (18 MΩ) used throughout the research. Filtered sea water (FSW) was obtained by means of filtering natural sea water (northern Adriatic Sea; salinity 38.1, pH 8.1) through Whatman 0.2 μm pore membrane filters (GE Healthcare Life Sciences, Little Chalfont, UK).

3.2 Synthesis of silver nanoparticles

AgNP were produced by the sodium citrate reduction method. Initially 21.2 mg of AgNO3 was diluted in 125 mL ultrapure water. The solution was heated until it began to boil and mixed with a magnetic stirrer. A 1% tri-sodium citrate aqueous solution was prepared by dissolving 0.5 g tri-sodium citrate into 50 mL of ultrapure water. Afterwards, 5 mL of this 1% solution was added to the boiling $AgNO_3$ solution, and the mixture was held at 100^oC until the color became pale yellow. The mixture was cooled to room temperature and was calculated to have a silver concentration of 100 mg/L.

The mechanism of the redox reaction is as follows:

$$4Ag^+ + C_6H_5O_7Na_3 + 2H_2O \rightarrow 4Ag + C_6H_5O_7H_3 + 3Na^+ + H^+ + O_2 (\uparrow)$$

3.3 Characterization of AgNP in ultrapure water with UV-Vis spectroscopy

The sample for UV-Vis spectroscopy was prepared as follows. Previously prepared stock AgNP solution was diluted in ultrapure water to give final concentrations of 1 and 10 mg/ L. All three solutions (1, 10 and stock 100 mg/ L) were placed into a quartz glass cuvettes with an optical path length of 10 mm. The

samples UV-Visible data was collected on a Shimadzu UV-1800 spectrophotometer with a double beam configuration. The wavelength spectrum ranged from 300 - 800 nm with a resolution of 1 nm. The collected data was processed with UVProbe 2.3.1 software (Shimadzu, Kyoto, Japan).

3.4 Sea urchin embryonal development test- sperm pretreatment

Sea urchin species *A. lixula,* also known as the black sea urchin, had been collected in Pula, Croatia (Valkane bay, July 2016). Samples were transported to Rovinj (40 km from Pula) to the Center for Marine Research of the Ruđer Bošković Institute.

Afterwards they were kept for several days in outdoor stone tanks with a natural sea water flow through system. In the morning the experiment was started, 5 L of fresh sea water was filtered through a 0.2 µm filter. Afterwards several sea urchins (*A.lixula*) were carefully collected (to avoid gametes being expelled due to stress) and put into smaller containers with fresh sea water and transported into the research facility.

Gametes were collected as formerly explained in Quiniou et al. (1999), with minor modifications. 0.5 M KCl solution was prepared by adding 3.73 g KCl into 100 mL of distilled water. Afterwards the sea urchins were shaken for a few seconds to induce the release of gametes, if nothing happened, 1 mL of 0.5 M KCl solution was injected through the peri-oral membrane and the sea urchins were shaken again, and as a consequence gametes were released.

Gametes were gathered from several individuals, with sperm being collected ‘dry’ with a pipette and afterwards placed in a PCR tube and held on ice (0 ^{0}C).

Eggs were carefully gathered into a small conical container with fresh sea water and left for a few minutes for settling to take place. Excess water from the container was removed. Subsequently the maturity of gametes was checked with an optical microscope. The criteria were: spherical eggs and mobile sperm. The used *A. lixula* were later released back to the sea at the site in which they were found (Valkane, Pula, Croatia).

Stock solution 1 was diluted (100 mg/ AgNP (synthesis has been explained in chapter 5.2.) tenfold, by diluting 1 mL of stock 1 (100 mg / L) into 9 mL of ultrapure water., giving stock 2, a silver nanoparticle solution of 10 mg / L. This process has been done for practicality reasons involving the next step.

The following quantities were then added to polystyrene cups: 100 uL of stock 1 (100 mg / L) to the “1000 µg / L” named cup, 50 µL of stock 1 into a “500 µg/ L” labeled cup, 100 µL of stock 2 (10 mg / L) within a “100 µg / L” tagged cup, 50 µL of stock 2 into the “50 µg / L” labeled cup, 10 µL of stock 2 to the “10 µg / L” named cup and 1 µL of stock 2 within a “1 µg / L” labeled cup. 100 µL of ultrapure water was added to the “control” labeled cup. 10 mL of filtered sea water was added to all cups giving a series of different concentrations within the cups. Afterwards to every silver nanoparticle solution of various concentration 100 µL of pure *A. lixula* sperm (held till now into PCR tubes on 0 ^{0}C) was added.

The solutions of *A. lixula* sperm and different concentrations of silver nanoparticle solutions were left at room temperature for 10 minutes. Then, 100 µL of the first mixture (1000 µg / L silver nanoparticle solution with 100 uL of pure *A. lixula* sperm) was put into six well plates. This step was repeated with the other mixtures (to avoid subjectivity, and confusion in the counting process the well plates with different silver nanoparticle concentrations were keyed). 6 parallels of 7 different silver nanoparticles concentration and 100 µL of pure *A. lixula* sperm (42 in total) were tested.

Afterwards 10 mL of filtered sea water and 40 µL concentrated egg cells (due deposition) were added into all well plates. The well plates were gently stirred to encourage fertilization and left at room temperature for 2 h. After this period, the embryos had reached the morula stage. At this point, the capability of sperm treated with various concentrations of silver nanoparticles to fertilize the egg cells was determined. This process was followed by inverted microscopy, and 100 egg cells of every replicate were randomly selected and determined if fertilization had occurred. Fertilized eggs had 1 or more grooves due to cell division, while in unfertilized eggs such a structure was not found.

After all data has been collected the embryos were held at 20^{o}C for 72 h with natural day – night cycle with occasional gentle agitation of the well plates. After 72h the fertilized eggs had reached the pluteus or larvae stage. Subsequently 100 larvae

from 4 replicates (of every silver nanoparticle concentration) were randomly chosen and checked for abnormalities by microscope. The samples were separated in four major groups with the following criteria, and shown in Figure 2.

The first group, also called control, had normally developed larvae with 4 fully developed extremities (front are shorter, so called “arms” and the rear are longer, called “legs”), gastrointestinal tract and characteristic cone shape. Members of the second group, or retarded larvae, were no more than half the size of the first group, or had undeveloped / deformed extremities (most common deformation found was a shorter extremity). The dead larvae or plutei were placed in the third group. Dead samples had started decaying or only the skeletal structure remained. The fourth group contained the undeveloped larvae. Unlike the second group (retarded larvae) the undeveloped larvae had completely fallen behind in the development process. Often no skeletal, gastrointestinal structure or extremities could be found since samples were still in the blastula or gastrula stage. Non-fertilized eggs were not counted.

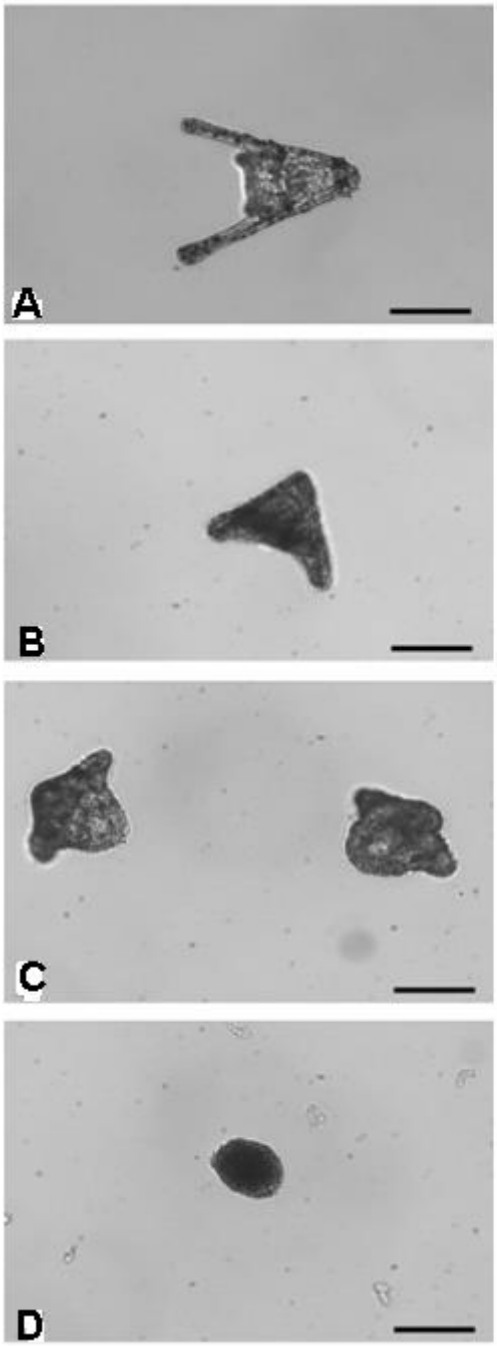

Fig. 2. Criteria by Burić et al.(2015), normal (A), retarded (B and C) and undeveloped *A. lixula* larvae (D)

4. Results

4.1 AgNP characterisation

The absorbance graph of the AgNP in ultrapure water stock solution is shown in Figure 3. On the x-axis the wavelength expressed in nanometers (nm), while on the y-axis the absorbance (expressed in absorbance units) of the stock solution is given. The green line shows the absorbance of the 100 mg/L AgNP stock solution, the blue line shows the absorbance of the same solution after 10x dilution (10 mg/L) and red line after 100x dilution (1 mg/L). This strong absorbance peak whose intensity varies linearly with concentration is assigned to the surface plasmon resonance (SPR), a collective resonant oscillation of surface electrons interacting with the electromagnetic field (light), of AgNP. The maximum absorbance is at 435 nm and this SPR wavelength indicates that the nanoparticles have a size of 60 nm (Gicheva and Yordanov, 2013).

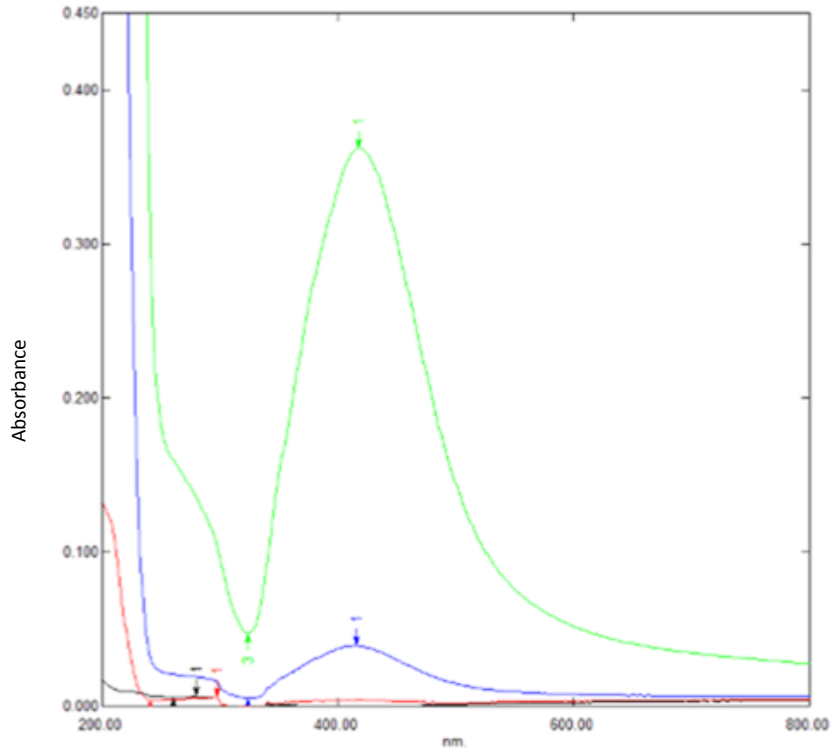

Figure 3. Absorbance spectra of as-synthesized silver nanoparticles (green line, 100 mg/L; blue line, 10 mg/L; and red line, 1 mg/L AgNP)

4.2 AgNP effects on the sea urchins sperm fertilization proficiency

The relationship between AgNP concentration and fertilization rates was determined based on the beginning of cell division in eggs, and is shown in Figure 4. For control samples where sperm had not been exposed to AgNP, i.e. denoted as 0 µg/L AgNP in Figure 4, the fertilization rate was found to be approximately 90%. However as the concentration of AgNP to which the sperm were exposed was increased to 50 µg/L there was a rapid decrease of fertilization success to 67%. Further incremental increases of AgNP concentration showed a linear decrease in fertilization rate. At 500 µg/L the fertilization rate was found to be 46%, while for an AgNP concentration of 1000 µg/ L only 33% of eggs showed the beginning of cell division.

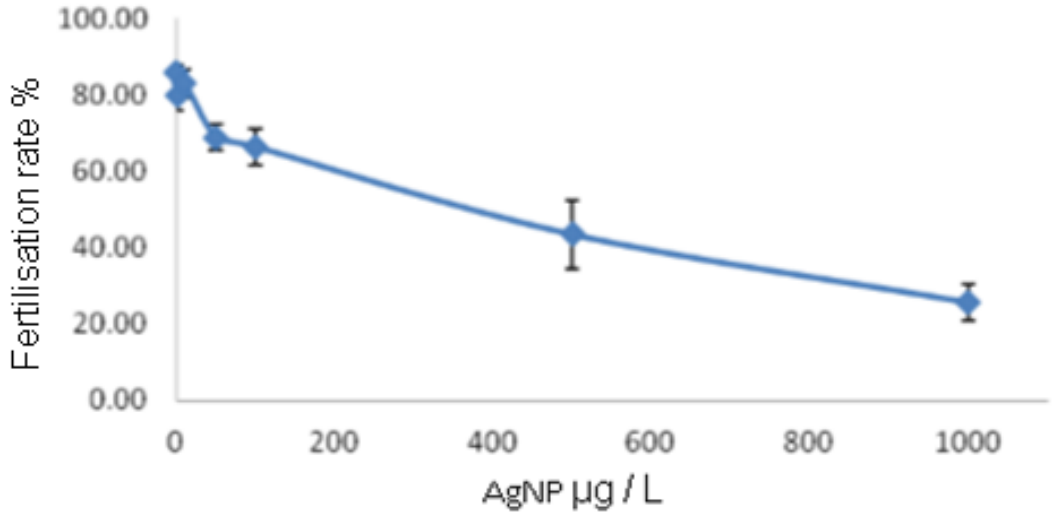

Figure 4. Relationship between AgNP concentration for sperm pre-treatment and fertilization rate in *A. lixula* eggs. Error bars indicate the standard deviation.

4.3 Effects on the pluteus stage in embryos fertilized with AgNP treated sperm

For those eggs which were successfully fertilized by AgNP-treated sperm, the embryonal development was tracked until the larvae reached the pluteus stage and the percentage of plutei which were determined to be normal, retarded, undeveloped and dead are shown in Figure 5. In the control group 95% of the larvae were found to be normally developed, while those sperm pre-treated with AgNP concentrations ranging from 1 to 100 µg/L showed a slight decrease of normal larvae to 88% and an increase of dead larvae to 7%. At the higher sperm pre-treatment concentrations of 500 µg/L and 1000 µg/L AgNP the percentage of normally developed larvae decreased to 77% and 72% respectively, with a corresponding increase in the number of larvae which were dead or showed arrested or retarded development.

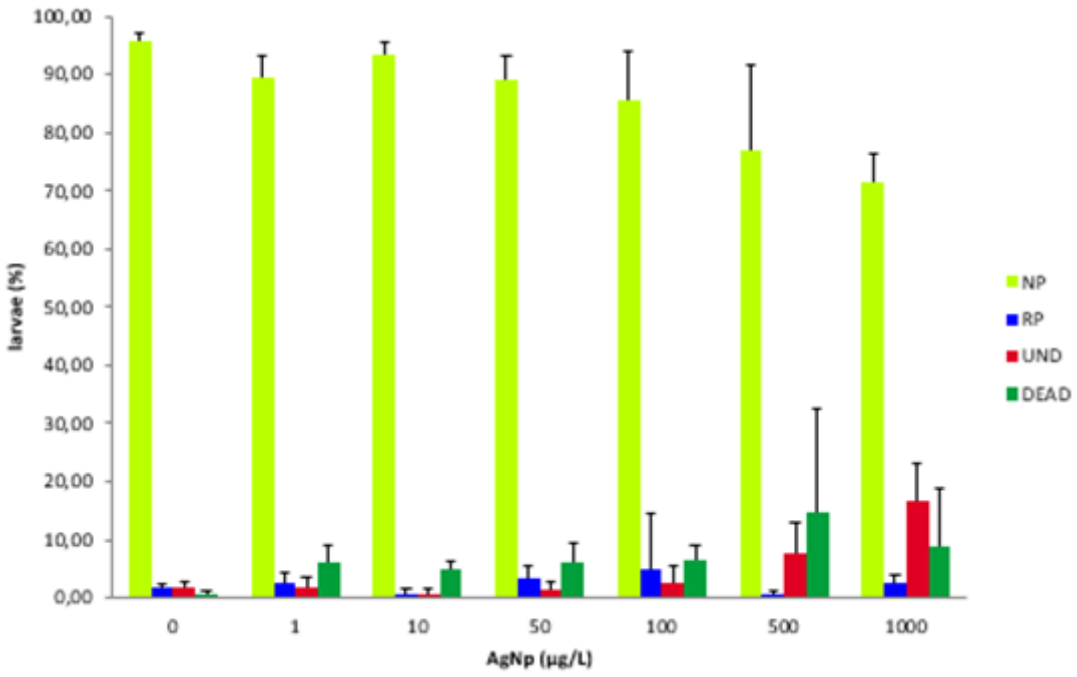

Figure 5. Percentage of normal (NP), retarded (RP), undeveloped (UND) and dead (DEAD) *A. lixula* larvae at various AgNP sperm pre-treatment concentrations

5. Discussion

Over the past number of years there is an increasing number of studies that have focused on determining the effects of metal nanoparticles on freshwater and brackish or marine organisms. In particular, many of these studies have investigated the impact of AgNP on a wide range of organisms and the effect of Ag^+ ions which are released from AgNP surfaces as they undergo oxidative dissolution in the aqueous environment. It has been shown that AgNP may induce in sea urchin larvae various negative effects such as, for example, fertilization failure, delay in development, stress, skeletal and gastrointestinal deformation (Burić et al., 2015). While Ag^+ ions are widely believed to be the primary cause of AgNP toxicity to orgamisms, published data on sea urchins seem to indicate that the nanoparticles themselves may be more detrimental to embryo development than Ag^+ ions (Šiller et al., 2013; Burić et al., 2015).

A.lixula was chosen for this experimental work since previous studies carried out on sea urchin larvae indicated that A. lixula maybe considered the most appropriate urchin species in terms of biomonitoring as it showed a higher sensitivity to silver nanoparticles than other Mediterranean urchin species, *viz. P. lividus* and *S. granularis* (Burić et al., 2015). For example, toxic effects from AgNP were noted for *A. lixula* at concentrations as low as 1 µg/L while embryos exposed to just 10 µg/ L of AgNP 30 min post-fertilization induced a decrease of normally developed larvae from 77.7% to 47.7%, (using the criteria reported by Carballeira et al. (2012)), and retarded larvae increased from 14% to 37.3%. Increasing the dose to 50 µg/L or 100 µg/L showed further negative impacts with complete stoppage in embryo development at all times of exposure post-fertilization. In the same study *P.lividus* embryos were also exposed to various concentrations of AgNP (1, 5, 10, 25, 50, 100 µg/L) at different developmental stages post-fertilization (30 min, 90 min, 6 h, 24 h). For AgNP concentrations up to 10 µg / L the development was unaffected while a dose of 50 µg/L gave significantly higher percentages of retarded and undeveloped larvae compared to control values - exposure 30 min post-fertilization gave only 50.7% normally developed larvae and 41.3% retarded larvae after three days.

However *A. lixula* larvae from AgNP pre-treated sperm (this work) or egg cells (N. Peruško, *personal communication*) showed less retardation and a lower

undeveloped larvae rate when compared to larvae treated with AgNP at various times post-fertilization (Burić et al., 2015). Further, in the present work, it has been shown that sperm`s ability to fertilize eggs was severely lowered only when they were pre-treated with AgNP concentrations exceeding 100 µg/L, with an essentially linear concentration-dependent decrease in fertilization success.

Pre-treatment of *A. lixula* eggs with similar concentrations of AgNP was carried out in a comparable experiment to the present work (N. Perusko, *personal communication*) with the former showing both similarities and differences to the data reported herein. Specifically, *A. lixula* eggs treated with AgNP showed a greater sensitivity to AgNP than sperm when fertilization rates are taken into consideration. For example, the sperm pre-treatment, even with a ten-fold greater concentration of AgNP (500 µg/L), showed 10% higher fertilization success than for pre-treated eggs at an AgNP concentration of 50 µg/L. This may indicate that pre-treated sperm are more robust in terms of being able to achieve fertilization compared to pre-treated eggs which may be more susceptible to the harmful effects of AgNP.

In broad terms, why pre-treated sperm show a decreased ability to successfully fertilize eggs may be related to several factors. One of these is that due to the sperm's smaller size, therefore a higher sensitivity to AgNP may exist for a given pre-treatment concentration with respect to egg pre-treatment. Moreover sperm mobility plays a key factor in the fertilization process. AgNP could have detrimental effects in that regard as with decrease in sperm mobility the probability of successful fertilization also decreases. Furthermore sperm´s chemoreceptors may be damaged by AgNP or the Ag^{+} ions that are inevitably released by the nanoparticles. However more in depth research with tailored experiments to address these points need to be carried out to clarify this process.

A second aspect which needs to be addressed is the Trojan horse mechanism of AgNP introduction into the egg with pre-treated sperm as the carrier. It has recently been reported that silver nanoparticles can be internalized into sperm cells (Yoisungnern et. al., 2015). It may be possible that a similar process may manifest in the egg cells. Due to the sheer size difference, less silver nanoparticles can be internalized into the sperm cell. Therefore when an AgNP pre-treated sperm cell fertilizes an untreated egg cell the nanoparticles get "diluted" in the sizable egg cell volume. On the other hand when an AgNP pre-treated egg cell, assuming that AgNP

have been internalized, is fertilized by an untreated sperm cell the dilution of AgNP load can`t occur. Accordingly, this may point to one of a number of mechanisms that may explain why detrimental results are found more for larvae derived from AgNP pre-treated egg cells. Thus, it is possible that internalized AgNP may play a significant role in sperm and egg cell toxicity, not only from a physical standpoint related to nanoparticles specific size and its ability to disrupt membranes and be internalized, but also from the chemical perspective as direct suppliers of Ag^+ ions inside sperm and egg cells.

With regards to embryonal development after fertilization, in both the non-pre-treated sperm and egg control groups around 90% of the larvae were normal. Increasing the AgNP dose concentration to 50 µg/L showed a slight decrease of normal larvae to 88% and increase of dead larvae to 7% when sperm pre-treatment was carried out. This indicates that, up to such concentrations, no significant negative effects were apparent with similar normally developed larvae to the control experiments. At high concentrations of 500 µg/L AgNP, only 77% larvae were normal. However, in a parallel experiment (larvae from egg cell pre-treatment; N. Peruško, *personal communication*) approximately 10 µg/L AgNP (i.e. a 50-times lower concentration than for sperm pre-treatment) was sufficient to decrease the normal larvae percentage to 77% and increase the retarded larvae to 19%. Severely detrimental results were noted at a concentration of 500 µg/L where only 40% of larvae were normal, 55% undeveloped and 5% retarded. This clearly shows that sensitivity to AgNP is not uniform across different cell types.

6. Conclusion

With the increasing range and volume of nanoparticles being produced every year, and their ability to eventually enter the environmental, investigations into the negative effects of nanopartilces on living organisms are of growing importance. In this work it has been shown that sperm cells have decreased fertilization success after they come in contact with silver nanoparticles in a concentration dependent manner. Further, normal larval development was also found to be affected, with an increasing percentage of those eggs that had been successfully fertilized showing growth retardation, arrested development or death. Based on these results, further research is required to uncover the exact mode of interaction of the nanoparticles with sperm, their possible internalization and how they impact on key pathways during embryonal developing of sea urchins.

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TEMELJNA DOKUMENTACIJASKA KARTICA

Sveučilište Jurja Dobrile u Puli Završni rad
Sveučilišni preddiplomski studij Znanost o moru

Sinteza i utjecaj nanočestica srebra na sperme morskog ježinca *Arbacia lixula*

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Sažetak

Primjena umjetno proizvedenih nanočestica raste svake godine u širokom rasponu područja kao što su: medicina, kozmetika, proizvodnja hardverskih dijelova, poljoprivreda, prehrambeni dodatci, itd. Na taj se način povećava i vjerojatnost da će nanočestice završiti u različitim dijelovima okoliša, poput: slatkovodnih sustava, tla, estuarija ili morske vode. Danas se nanočestice srebra koriste kao: optički pojačivači, toplinski i električni vodiči, antibakterijski premazi, biosenzori itd. Upravo zbog toga, studije o njihovoj okolišnoj sudbini, uzrocima toksičnosti, bioakumulaciji i upijanju, od ključne su važnosti kako bi se spriječili negativni učinci na ljude i ostale organizme. U ovom radu ispitan je utjecaj srebrnih nanočestica na spermu morskog ježinca *Arbacia lixula*. Proizvedene su srebrne nanočestice (AgNP) određenog promjera (60 nm). Nakon toga, sperma je bila izložena različitim koncentracijama AgNP (od 1-1000 µg/ L), te se pratilo da li tretirana sperma može oploditi jajne stanice i koji je postotak larvi bio normalno razvijen, zaostao, nerazvijeni embrii ili mrtvi. Stopa smrtnosti znatno je smanjena na 67% sa 50 ug / L AgNP (90% kontrola). Daljnji porast koncentracije AgNP pokazuje linearno smanjenje stope oplodnje. U pogledu razvoja larvi, linearno smanjenje normalnih larvi primiječeno je kod koncentracije većoj od 50 µg/ L. Stopa oplodnje bila je niža u tretmanu sperme u odnosu na objavljeni podatci za jajne stanice izlozeni srebrnim nanočesticima. Međutim, broj normalno razvijenih larvi bio je viši kod predtretmana sperme s AgNP. Mogući razlozi jesu: ulazak AgNP u jajne stanice te moguće razrijeđenje tijekom oplodnje. Potrebna su daljnja istraživanja o ulasku AgNP u jajne stanice te njihovom utjecaju na spermu ježinaca.

Ključne riječi: Nanočestice srebra, *Arbacia lixula,* Uspješnost oplodnje, Eksperiment embrionalnog razvoja morskog ježinca

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Datum obrane:

BASIC DOCUMENTATION CARD

Juraj Dobrila University of Pula Bachelor thesis

University undergraduate study programme – Marine Sciences

Synthesis of silver nanoparticles and their impact on the sperm of sea urchin *Arbacia lixula*

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ABSTRACT

As the use of synthesized nanoparticles grows every year in a broad range of fields like: medicine, cosmetics, hardware parts manufacturing, agriculture, dietary supplements, etc. So does the possibility that such nanoparticles finish into various environmental media such as freshwater systems, soil, estuarine or marine waters. Recently, silver nanoparticles have been used as optical enhancers, thermal and electrical conductors, antibacterial coatings, biosensors, etc. Therefore studies about their environmental fate, toxicity patterns, bioaccumulation and uptake are of pivotal importance to prevent negative effects on humans and other keystone species. In this study, the impact of silver nanoparticles on the sperm of sea urchin species *Arbacia lixula* has been investigated. Silver nanoparticles (AgNP) of a certain diameter have been produced (60 nm). Subsequently *A. lixula* sperm were exposed to various concentrations of AgNP (from 1-1000 µg/ L) and observed if treated *A. lixula* sperm was able to fertilize the egg and what percentage of the plutei were normal, retarded, undeveloped or dead. Fertilization rate was significantly lowered to 67% at 50 µg/ L of AgNP (90 % control). Further increment of AgNP concentration shows a linear decrease in fertilization rates. In regards to larval development major effects and a linear reduction of normal larvae were identified at concentrations exceeding 50 µg/L. Fertilization rates were lower for sperm treatment than literature values for urchin eggs treated with silver nanoparticles. However normal larvae were more frequent after sperm treatment, and may be due to internalization of AgNP and a dilution effect when fertilization takes place. Further investigation of AgNP internalization and the effects this process is highly needed.

Key words: Silver nanoparticles, *Arbacia lixula,* Fertilization rate, Sea urchin embryo development test

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Thesis defense: