Universidade de Aveiro Departamento de Biologia

Ano 2015

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Toxicity of silver nanoparticles and silver nitrate on *Nassarius reticulatus* larvae

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro, Investigadora Auxiliar do Centro de Estudos do Ambiente e do Mar (CESAM) laboratório associado da Universidade de Aveiro (UA), e coorientação da Doutora Susana Galante-Oliveira, Investigadora em Pós-Doutoramento do CESAM-UA.

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palavras-chave Nanotecnologia, Nanopartículas de Ag, *N. reticulatus*.

resumo

A produção e utilização de nanopartículas de prata (AgNPs) em diversas aplicações têm crescido rapidamente, principalmente devido à sua atividade antibacteriana. A inclusão de AgNPs em muitos produtos de consumo conduziu a um aumento da sua libertação no meio ambiente, especialmente nos ecossistemas aquáticos.

As AgNPs atingem tanto o ambiente marinho como o de água doce a partir da descarga de efluentes de estações de tratamento de águas residuais, apresentando comportamento diferenciado nestes dois meios, potencialmente influenciando a sua toxicidade. O estudo da toxicidade das AgNPs em organismos marinhos é extremamente importante na avaliação do potencial risco da presença de AgNPs no ambiente.

A toxicidade de AgNPs emorganismos vivos é dependente de várias condições ambientais. No que se refere à toxicidade das AgNPs em ambiente marinho, verifica-se uma lacuna de informação relativamente aos efeitos tóxicos a diferentes salinidades.

Este trabalho surge para preencher esta lacuna, sendo o primeiro relato do efeito de AgNPs em larvas de gastrópodes marinhos, usando *Nassarius reticulatus* como caso de estudo. Foram colhidos adultos de *N. reticulatus* de uma população de referência da Ria de Aveiro (NW Portugal, 40° 38' 33.24"N | 8° 44' 06.69''W). Os espécimes foram transportados para o laboratório em água do local e aí mantidos em aquário até à postura de cápsulas ovígeras. As cápsulas foram mantidas até à observação de larvas velígeras no seu interior, cuja eclosão foi induzida por cesariana. Estas larvas recém-eclodidas foram então expostas a concentrações nominais de AgNPs e Ag^{+} (0,1, 1, 10, 100 ug de Ag / L), durante 96 h, na presença ou ausência de alimento.

A mortalidade larvar e o comportamento de natação, nomeadamente a inibição do batimento do velum, foram determinados para cada um dos tratamentos.

A concentração letal média (CL50) das AgNPs revelou-se superior à da Ag iónica ($AgNO_3$). Os resultados também revelaram que o impacto negativo das AgNPs na natação das velígeras de *N. reticulatus* é superior (EC50-96 h 0.044 µg Ag/L) quando comparado com o efeito da Ag iónica (EC50-96 h 1.044 µg Ag/L). Contudo, embora a inibição da movimentação do velum das larvas ter diminuido significativamente na presença de Ag^{+}, as AgNPs não mostraram quaisquer efeitos na inibição do batimento do velum. Adicionalmente, a presença de alimento revelou ser um fator importante, podendo causar uma redução significativa na mortalidade das larvas de *N. reticulatus* expostas a AgNPs.

keywords Nanotechnology, Silver nanoparticles*, N. reticulatus*.

abstract

Production and utilization of silver nanoparticles (AgNPs) for various applications is growing rapidly, mainly due to their antibacterial activity. Their inclusion in many consumer products led to an increased release of AgNPs in the environment, especially in aquatic ecosystems.

AgNPs reach both freshwater and marine environments from the effluents of the wastewater treatment plants, presenting differentiated behavior in these two environments potentially influencing its toxicity. The study of AgNPs toxicity to marine organisms is extremely important to the assessment of the potential risk of AgNPs in the environment.

The toxicity of AgNPs on the living organisms is dependent on various environmental conditions. Regarding the toxicity of AgNPs in the marine environment, there is a lack of information on the toxic effects at different salinities.

This study upsurges to fill this gap, being the first report on the effects of AgNPs on marine gastropods, using *Nassarius reticulatus* as a case study. *N. reticulatus* adults were collected from a reference population in Ria de Aveiro (NW Portugal, 40° 38' 33.24"N | 8° 44' 06.69''W). Specimens were transported to the laboratory in local seawater and kept in aquaria to spawn. Egg capsules were maintained until veliger larva were noticed, which enclosure was induced by cesarean. These recently hatched larvae were then exposed to nominal concentrations of AgNPs and Ag^{+} (0.1, 1, 10, 100 µg Ag/L) for up to 96 h, either in the presence or absence of food.

Larval mortality and swimming behavior –namely the velum beating arrest– were determined for each treatment. The median lethal concentration (LC50) of AgNPs was higher to that of ionic Ag ($AgNO_3$). Results also revealed that the negative impact of AgNPs on *N. reticulatus* veligers swimming ability is higher when compared with the effect of ionic Ag (EC50-96 h 1.044 µg Ag/L). However, although the velum arrests have significantly decreased under Ag^{+} exposure, AgNPs did not show any effects. Additionally, the presence of the food proved to be an effective factor that can cause a significant drop in the mortality of the *N. reticulatus* larvae exposed to AgNPs.

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Chapter 1

General Introduction

1. General introduction

1.1 Introduction

Engineered nanoparticles (NPs) have been increasingly used, with various applications in different scientific and industrial fields, because of their unique properties. However, the application of such novel materials is highly dependent on their specific properties. In one hand, silver nanoparticles (AgNPs) present high electrical and thermal conductivity, catalytic activity, and chemical stability; on the other hand, they can be satisfactory applied as antibacterial agents in the various consumer products (Fabrega, Luoma, Tyler, Galloway, & Lead, 2011; Gomes, Pereira, Cardoso, & Bebianno, 2013). The food industry (see e.g., Cushen et al. 2013), water treatment, clothing, and the production of the softeners, soft toys, kitchen utensils, computer keyboards, baby products (Shaw & Handy 2011), and dental materials (Allaker & Memarzadeh, 2014) are some examples of the application of AgNPs motivated by their multiple physico-chemical properties.

Nevertheless, and despite its unquestionable usefulness, the application of these novel materials has been pointed as a main source of AgNPs to the environment, leading to an increase of its environmental concentration. Consequently, environmental and health concerns have been raised, particularly regarding the complex reactions involving this type of nanoparticles (Hu, Wang, Wang, & Wang, 2012; Oberdörster, Oberdörster, & Oberdörster, 2005). Permanent bluish-grey discoloration of the skin (argyria) and eyes (argyrosis) after humans chronic exposure (see e.g., Panyala et al. 2008), in vitro toxic effects on human lymphocytes, *in vivo* toxicity on the *Allium cepa* and *Nicotiana tabacum,* and toxic effects on the Swiss albino male mice (see e.g., Ghosh et al. 2012) are just some examples indicating AgNPs toxicity on the living organisms.

Considering the ongoing applications of the AgNPs, the scientific awareness on subsequent toxic effects has been considerably increased during the recent years. Geranio et al. (2009) reported that about 0.3-377 μg Ag/g textile could be released from socks during washing. They believe that this amount is highly dependent on the techniques applied for the AgNPs utilization in the textile structure, the synergistic effects of chemical agents, and the mechanical stresses applied to the textile under washing process. Gottschalk et al. (2009) concluded that, in sewage treatment plant effluents, the aquatic organisms are at risk due to the toxic effect of AgNPs. The severity of the associated risks seems to be directly related to the amount of AgNPs released to

the receiving environments. The results of a simulated study carried out by Dumont et al. (2015) showed that AgNPs can reach concentrations even greater than 0.3 ng/L in 10% of most of the Eastern and Southern European rivers monitored.

Moreover, processes of dissolution and agglomeration of Ag nanoparticles (Fig. 1.1) have been shown to have an important effect on their toxic effects. Ionic species released from NPs may be toxic, thus dissolution process need to be considered when studying NPs toxicity. Dissolution occurs when an ion detaches from the particle and migrates into the solution (Borm et al., 2006) while aggregation is controlled by attractive and repulsive forces on the particles and will have a great influence on their sedimentation (Keller et al., 2010). Based on a model developed by Markus et al. (2015), AgNPs tend to homo-aggregate in lab-scale studies, while in the real-scale observations, hetero-aggregation is the main behavior of NPs, which normally occurs as a result of the adsorption of such fine particles to other types of particles. As an example, aggregations of AgNPs with other organic compounds may change their bioavailability (Farré, Gajda-Schrantz, Kantiani, & Barceló, 2009) and, as a consequence, their toxicity.

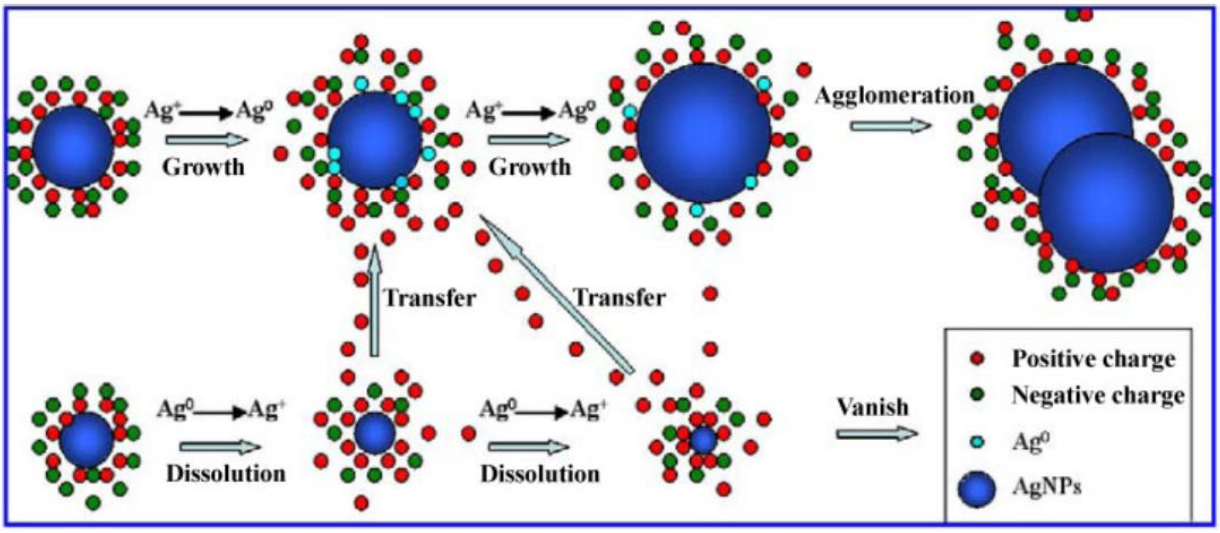

Figure 1.1. The dissolution and agglomeration process in effect of the surface charge change with the augmentation of particle size for AgNPs (Shi, Ma, Xu, Zhang, & Yu, 2012).

The environmental conditions under which the AgNPs are present influence their fate and toxic effects. In this regard, the mobility and behavior of AgNPs in aquatic systems can be directly affected by some influencing factors. Interactions of the NPs with biota (see Bottero et al. 2011), NP properties (e.g. size, shape, surface coating, surface charge (see Fig. 1.1), and the existence of the light (Navarro, Baun, et al., 2008; Sharma, Siskova, Zboril, & Gardea-Torresdey, 2014) are

among the main important measures that should be taken into consideration when studying the life cycle and fate of AgNPs (Kamali, Gomes, & Khodaparast, 2014).

1.2 Fate of AgNPs in aquatic environments

Detection of the AgNPs in the environment is considered one of the main steps for the evaluation of the real-scale toxic effects of these particles. However, detecting the engineered nanomaterials in the environment can be difficult, and sometimes simply impossible by the currently available methods (Hassellöv, Readman, Ranville, & Tiede, 2008). Moreover, the behavior of such particles after being released into the environment seems to be highly dependent on various factors such as the NPs characteristics and the media properties. The salinity of the aquatic media is one of the main factors influencing NPs behavior in the environment (Chinnapongse, MacCuspie, & Hackley, 2011). Therefore, in the following parts, a literature review has been provided on the fate and behavior of AgNPs in freshwater and marine environments.

1.2.1 AgNPs in freshwaters

The lifetime of AgNPs in the environment is probably short, because of its rapid oxidation and the strong tendency of Ag to link with O, Cl, S, and organic compounds, especially those containing thiol groups (Levard, Hotze, Lowry, & Brown, 2012) and, as the binding of sulphide to AgNPs produces the very stable Ag_2S, the toxicity of AgNPs can be reduced (O. Choi et al., 2009), (Fig. 1.2).

Toxicity of AgNPs can be altered in different water chemistry conditions. For instance, the results of a study by Liu et al. (2014) indicated that the natural organic matter (e.g., humic acid, bovine serum albumin, and alginic acid) had no significant impact on AgNPs stability and, thus, on their toxicity on bactericidal activity. But, by the addition of Ca^{2+} in the media, AgNPs stability and toxicity decreased significantly, possibly because of the NPs aggregation enforced in this situation. Moreover, chelating agents can play an important role on the toxicity of AgNPs. Ethylenediaminetetraacetic acid (EDTA) can display a concentration-dependent impact on the AgNPs toxicity: at low concentration (1 mg/L), EDTA reduces the AgNPs toxicity by conversion of Ag^+ and Ag–Cl complex into lower toxic Ag–EDTA complex; however, at higher concentrations (5 and 20 mg/L), EDTA increases AgNPs toxicity, possibly due to the destruction of cell membrane structures, raising the entry of AgNPs and Ag^+ species into bacteria cells (X. Liu et al., 2014). The chronic exposure of *Ceriodaphnia dubia*

to Ag^+ revealed that dissolved organic carbon (0.4 mg/L) decreased Ag ions toxicity in 50% while, by adding sulphide (75.4 nM), the toxicity was reduced to 42% (Naddy et al., 2007).

Some studies have investigated the effects of certain environmental conditions on the assessment of AgNPs toxicity in aquatic living systems. For example, Bian et al. (2013) studied the impact of Ag^+ from AgNPs in the aquatic macrophyte *Lemna gibba* under various concentrations of nitrogen (N) and phosphorus (P). The authors reported a lowest observed effect concentration (LOEC) of 2µg/L, but after reducing N and P concentrations, increased N:P ratios and LOEC values were registered. Also, the determination of AgNPs stability in OECD media (used for toxicity tests in *D. magna*) rendered a very fast aggregation behavior; however, when diluting the media by a factor of 2, 5, or 10, AgNPs aggregation was reduced without affecting the viability of *D. magna* (Römer et al., 2011).

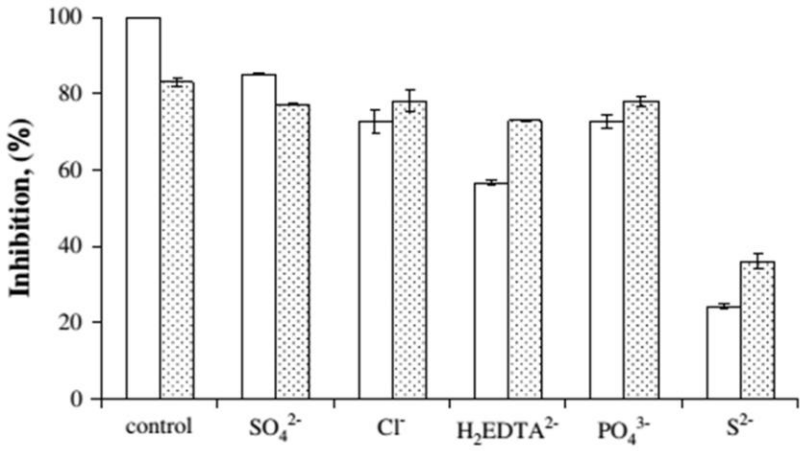

Figure 1.2. Impact of different ligands (at 10 µM) on decreasing AgNPs (open column) and Ag^+ (dotted column) toxicity at 1 mg/L (O. Choi et al., 2009). Toxicity was assessed as the % of inhibition of microbial activity.

Dissolved oxygen and protons are needed for the oxidation reactions that release Ag^+ from AgNPs. The rate of ion release can be enhanced through temperature elevation (0-37°C), and can be dropped by increasing pH or by the addition of humic or folic acids (J. Liu & Hurt, 2010). Although in the environment, under high concentration of dissolved oxygen, AgNPs cannot persist as individual particles due to the transformation to Ag^+; however, the slow rate of the oxidation process may provide enough time for the AgNPs to reach biological targets by various pathways such as ingestion or endocytic/phagocytic activity (J. Liu & Hurt, 2010).

1.2.2 Ag NPs in marine waters

Changing the physicochemical conditions such as temperature, salinity, and dissolved oxygen, can modify the configuration of AgNPs and alter their aggregation state. Thus, possibly due to the higher ionic strength of saltwater (in comparison to freshwater) AgNPs show several negative impacts and toxicity (Lapresta-Fernández, Fernández, & Blasco, 2012). Sodium, magnesium, calcium, and potassium cations and chlorides, sulphate, and bicarbonate anions, are the main ionic ingredients of seawater. Among them, the sodium and chloride concentrations are higher.

Stuart et al. (2013) reported that the complete aggregation of AgNPs in artificial seawater (salinity 31 psu, with 487 mM NaCl) occurred in 47 min. Moreover, it was also reported growth of AgNPs of 40 nm in artificial seawater and the formation of aggregates larger than 400 nm (Buffet et al., 2014; Bian et al., 2013). A study by Gomes, Araújo, et al. (2013) reported an acceleration in the formation of larger AgNPs in seawater (salinity 36.3 psu), particles' size ranging from 97 to 690 nm. Meyer et al. (2010) have also reported a remarkable aggregation of AgNPs (1-1.6 µm), and the precipitation of Ag^+, using K^+ medium. It should be noted that the K^+ medium –~5.4 parts per thousand (g/L) salts, mostly as NaCl and KCl– can be consider as "brackish" water, as it is about 1/6 the salinity of full-strength seawater –~35 psu or g/L salt, mostly NaCl. AgNPs aggregation and suspension stability in different salinities –deionized, estuarine (17 psu), and marine (33 psu) waters– during 7 days have also displayed increased aggregation at higher salinities (Khan et al., 2012).

Dai (2012) showed that the availability of silver in seawater is very low because of the reactions involving Ag ions and inorganic ligands (i.e., Cl^-), resulting in its sedimentation. The study of the persistence of citrate-capped 20 nm AgNPs in aquatic media indicated that, in saltwater with more than 20 mol/L sodium chloride, AgNPs were unstable, with high aggregation and sedimentation rates with increasing salinity (Chinnapongse, MacCuspie, & Hackley, 2011). The results of the investigation on the instability of carboxylate–AgNPs in the presence of Na^+ or K^+ revealed that the presence of AgNPs with salt (NaCl or KCl) could change the pH in the aqueous media (Pokhrel, Andersen, Rygiewicz, & Johnson, 2014). Changes in pH values were more pronounced in the case of NaCl, showing different interactions of Na^+ and K^+ with AgNPs in aqueous media (Pokhrel et al., 2014). In this regard, another study indicated that the impact of divalent cations –$CaCl_2$, $Ca(NO_3)_2$, $CaSO_4$, $MgCl_2$ and $MgSO_4$– on the aggregation of citrate-coated AgNPs is stronger than of monovalent

cations such as NaCl, $NaNO_3$, and Na_2SO_4 (Baalousha, Nur, Römer, Tejamaya, & Lead, 2013). Interestingly, the authors also found that, in the presence of chloride, AgCl NPs may be formed and sorb to the surface of cit-AgNPs, increasing NPs aggregation.

The physical properties of AgNPs can also be affected by salinity. The salinity increase can cause the AgNPs surface charge rise (García-Alonso et al., 2014). The addition of AgNPs to low salinity saltwater has been shown to have no major impact on the water clarity or visible sedimentation by Salari Joo et al. (2013), while in moderate and high salinities (6 and 12 psu, respectively) the authors observed the formation of black and brownish thin layers on the bottom of the aquarium after 48 h (most of sedimentation occurred during the first 4 h of the experiment).

Despite the numerous reports on the behavior of AgNPs in saltwater, data on AgNPs toxicity to marine species are scarce (Handy, Owen, & Valsami-Jones, 2008). There is a wide literature record on the negative impacts and toxicity for freshwater species. However, the behavior of AgNPs is proved to differ between freshwater and the marine environment (Handy et al., 2008) being impossible to use the data reported for freshwater to infer the toxicity in marine species. Even undergoing rapid aggregation and sedimentation in saltwater, AgNPs might be a threat for biological communities in marine ecosystems. AgNPs aggregate and sediment, but can also be trapped to the organic surface micro layers, thus posing risks to both benthic species and to zooplankton, including the early life stages of many organisms (Handy et al., 2008). AgNPs toxicity is particularly challenging for early life stages, since they may not be as resilient as the adults. Due to this vulnerability, and as recruitment cohorts are the foundation for any population success, the assessment of the toxicity of AgNPs to marine embryos and larvae is of primary importance (Matranga & Corsi, 2012).

1.3 Aims and thesis structure

As it was already reported, AgNPs can reach the marine environment and induce toxic effects on the living organisms. Thus, since the data on the toxicity of AgNPs in marine species are scarce, the present work aims to assess the toxic effects of AgNPs, with particle sizes of 3-8 nm, on early life stages of the gastropod *Nassarius reticulatus.* This marine gastropod is ubiquitously distributed in coastal areas of the North Atlantic. It is a scavenger (carrion feeder), and is well-known bio-indicator species of tributyltin (TBT) pollution.

Several mechanisms are involved in the toxicity of AgNPs towards the aquatic organisms. Although, as the swimming behavior is among the most relevant abilities for the

survival of free-swimming larval stages, the endpoints assessed in the current work included mortality, the swimming pattern, and the velum beating arrests. Moreover, these endpoints were assessed in larvae exposed to Ag^+ (as $AgNO_3$) and compared to that of larvae exposed to AgNPs. To present a condition near what normally happen in the aquatic environment, the toxicity of AgNPs was studied in the presence or absence of food.

Therefore, the present work is organized as follows:

- The first chapter presents the main concepts related to AgNPs such as information about their unique features, wide range of applications, and their various behaviours when discharged in freshwater and saltwater environments.

- A second chapter entitled “Toxicity of AgNPs in aqueous media” reviews the information available regarding the toxic effects of AgNPs on freshwater and seawater biological communities.

- The third chapter, “Toxic effects of silver nano-particles and silver nitrate on *Nassarius reticulatus* veliger”, presents the results of our study on the AgNPs and $AgNO_3$ toxicity on different endpoints in *N. reticulatus* larvae.

- The last chapter of this thesis summarizes the main conclusions and the final considerations that arose from this work.

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Chapter 2

Toxicity of AgNPs in aqueous media

2. Toxicity of AgNPs in aqueous media

2.1 Abstract

Production and utilization of silver nanoparticles (AgNPs) for various applications is growing rapidly. However, this material seems to have some ecological adverse effects. The migration of engineered nanoparticles (NPs), under most environmental conditions, can be restricted by some factors such as collision and contact with surfaces of porous media, ionic strength, and significant concentrations of calcium or magnesium together with agglomeration properties of AgNPs. Moreover, transportation of AgNPs may be limited by factors like pH, light, biota, etc. which can control the solubility and electrostatic interactions between these particles and the aquatic environments. AgNPs can be taken up by the biota and, subsequently, enter the cells causing oxidative stress, which can damage the cell membrane and subcellular components. It leads to the leakage of intracellular materials and, eventually, to cell death. Hence, the main goal of this chapter is to review the recent studies on the toxicity of AgNPs on the communities living in fresh and marine waters. These studies outcomes indicate that the toxicity of AgNPs on the living organisms is dependent on various environmental conditions, namely the medium salinity. In this regard, the amount of work necessary for evaluating the toxic effects of AgNPs, especially in marine environments and under different salinities is not satisfactory. Also there is a need for further studies to predict possible hazards associated with the presence of AgNPs in both fresh and marine environments.

Keywords: AgNPs, freshwater, marine environment, Toxicity.

2.2 Introduction

AgNPs are widely used in the nanotechnology industry and consumer products, especially due to their antibactericidal properties. The production of AgNPs has increased during the recent decade, being one of the most produced NPs (Meyer et al., 2009). On the other hand, the recycling of the products containing AgNPs has been counted as a main source of the AgNPs discharge in the environment (i.e., aquatic environments), leading to increase the subsequence environmental and health concerns, caused or induced through

the complex reactions involving such types of nano particles (Hu, Wang, Wang, & Wang, 2012; Oberdörster, Oberdörster, & Oberdörster, 2005). Thus, assessing their toxic effect to aquatic biological communities is extremely important.

Most of the studies on AgNPs toxicity have been conducted in freshwater for many different organisms, including both invertebrates (Croteau, Misra, Luoma, & Valsami-Jones, 2011; Jie Gao et al., 2012) and vertebrates (Aerle et al., 2013; Chio et al., 2012). However, there are not many studies reporting the toxicity of AgNPs in marine organisms. Moreover, as AgNPs behavior is considered to be completely different in freshwater compared to seawater (Levard, Hotze, Lowry, & Brown, 2012), there is a pressing need to report the negative effects of this type of NPs in marine species and under different environmental conditions, namely of salinity. In this way, the present review aims to summarize the available data related to the toxicity of AgNPs to aquatic organisms and the factors that may affect it.

2.3 Toxicity of AgNPs in freshwaters

So far some studies have been carried out to assess the toxic effects of AgNPs and ionic Ag in freshwater. Nevertheless, their outcomes differ between species and even in a same species. For example, Gao et al. (2015) showed that AgNPs are more toxic than $AgNO_3$ in zebra fish, *Danio rerio* (LC_{50} of 0.14 and 0.80 mg/L for AgNPs and $AgNO_{3,}$ respectively). However, reports on the lower toxicity of AgNPs when compared to Ag^+ are also available in the literature, as for example for the green algae *Chlamydomonas reinhardtii* (Navarro et al., 2008), the water flea *Daphnia magna* (Newton, Puppala, Kitchens, Colvin, & Klaine, 2013; Silva et al., 2014), and also for the zebra fish by Massarsky et al. (2013) and Yeo & Yoon (2009). When zebra fish embryos were exposed to AgNPs (20-30 nm) and Ag^+ at 10 and 20 ppt, higher mortality was registered in ion-exposed groups (Yeo & Yoon, 2009). Another study on the impact of AgNPs on zebra fish showed that Ag^+ was more toxic than AgNPs, with LC_{50} of 0.07 and 1.18 µg/mL for Ag^+ and AgNPs, respectively (Massarsky et al., 2013).

Conflicting results are also found in studies addressing the ion/nanoparticle effect caused by AgNPs exposures. Newton et al. (2013) showed that the toxicity of AgNPs to *Daphnia magna* is directly related to the amount of Ag^+, which is released from AgNPs. Furthermore, AgNPs toxicity was found to be not only related to the release of Ag^+ in *Raphidocelis subcapitat*, *Chydorus sphaericus*, and *Danio rerio* (Wang, Chen, Li, Shao, & Peijnenburg, 2012). Kennedy et al. (2010) suggested that acute toxicity of AgNPs to *Daphnia magna*,

Pimephales promelas, and *Pseudokirchneriella subcapitata* is mainly attributed to Ag^+ that its release from silver nanoparticles.

Nevertheless, reducing the concentration of Ag^+ released from AgNPs, even with the ion exchange treatment of AgNPs, did not change the toxicity of AgNPs for *D. magna* (Hoheisel et al. 2012). Accordingly, the toxicity of AgNPs is not only attributed to the release of Ag^+ that, at higher concentrations, delayed hatching in a similar way by both forms of Ag (Massarsky et al., 2013). The study by Navarro et al. (2008) on the AgNPs toxicity on the green algae *Chlamydomonas reinhardtii* showed that although Ag^+ can present 18 times more toxic effects than AgNPs, reduction of photosynthesis could not be explained by the Ag^+ released by AgNPs.

AgNPs behavior in the freshwater environment (e.g. dissolution, aggregation/agglomeration) is dependent on particles properties such as size, surface area, and coating material (Dams, Biswas, Olesiejuk, Fernandes, & Christofi, 2011; Fabrega, Luoma, et al., 2011). The results of a study on bacterium isolated from activated sludge showed that, based on the calculated LC_{50}, the smaller AgNPs are more toxic than those with greater particle size, due to the higher surface area, and it is dependent to the amount of Ag^+ that can be released from AgNPs (Dams et al., 2011). Hoheisel et al. (2012) showed the enhancing of toxicity to *Daphnia magna* with declining the nanoparticles size, with LC_{50} of various AgNPs sizes (10, 20, 30, and 50 nm) ranging from 4.31 to 30.36 mg/L. Accordingly, Seitz et al. (2015) showed a decline in AgNPs toxicity to *Daphnia magna* by increasing the size (20, 30, 60 and 100 nm). However, Li et al. (2010) found no difference in toxicity of AgNPs with various sizes (36, 52, and 66 nm) to *D. magna*, with the LC_{50} ranging from 3 to 4 µg/L. Moreover, acute and chronic exposures of *Daphnia magna*, *Cyprinus carpio* to Ag, with a similar mass dose of AgNPs and micro-sized silver, showed that AgNPs were more toxic than micro-sized silver (Gaiser et al., 2012).

The coating material of a nanoparticle's surface determines some of its properties, such as dissolution. To determine the influence of different coatings on the toxicity of AgNPs, Silva et al. (2014) exposed *Daphnia magna* to citrate-coated AgNPs, poly vinylpyrrolidone-coated (PVP) AgNPs, and branchedpolyethyleneimine-coated (BPEI) AgNPs. The toxicity of AgNPs was as the following order: BPEI AgNPs> citrate AgNPs >PVP AgNPs. When exposing organisms of three different trophic levels –*Raphidocelis subcapitat*, *Chydorus sphaericus*, and *Danio rerio*– to AgNPs –bare, PVP-coated, and monodispersed (DIS AgNPs)– Wang et al. (2012) showed that the toxicity of these AgNPs reduced in the order DIS>PVP>Bare for all

tested organisms. Both studies confirm that coating material is extremely important when assessing AgNPs toxicity.

The release of Ag^+ from AgNPs is also a medium-dependent reaction in freshwater (Wang et al., 2012). The presence of Suwannee River humic acid (SRHA) can linearly decrease the toxicity of AgNPs (20-30 nm) to *D. magna*, although no considerable effect on the release of Ag^+ from AgNPs was observed (Jie Gao et al., 2012). In agreement, the assessment of different factors such as pH (6.5 and 8.0), and dissolved organic matter (DOM; 0.1 or 8.0 mg total organic carbon/L) on acute and chronic toxicity of AgNPs to *D. magna* by Seitz et al. (2015) showed a decline in AgNPs toxicity by increasing pH, and DOM.

Shi et al. (2012) studied the effect of light on the toxicology of AgNPs on *Tetrahymena pyriformis*. The authors showed that Ag^+ toxicity is less than that of AgNPs under dark conditions, while the toxicity of AgNPs is reduced in an environment with light, fact attributed to the growth of AgNPs agglomerates by light irradiation. The AgNPs surface potential can be changed from negative to positive under light conditions, and so it can lead to serious agglomeration after 30 min of strong light irradiation, and large AgNPs (more than 1 mm) may settle. The dissolution rate of Ag^+ is higher for smaller AgNPs due to their higher energy than larger particles (Shi et al., 2012). By investigating different concentrations of humic acid (HA) and sunlight irradiation on AgNPs cytotoxicity on aquatic bacterial communities, Dasari & Hwang (2010) concluded that for determining the toxicity of AgNPs in the freshwater, case-by-case study may be necessary. A study on the dynamic changes of AgNPs showed that increasing the AgNPs dispersed in the media can result in the increase of AgNPs toxicity (Römer et al., 2013).

Navarro et al. (2008) indicated that, in addition to abiotic and chemical conditions, biotic interactions should be taken into consideration to determine the effect of AgNPs in the freshwater. By studying the effects of AgNPs on zebra fish embryos, *D. rerio*, Cunningham et al. (2013) showed that AgNPs toxicity to aquatic organisms is dependent on different factors, stressing as the key factors the capping agent, the zeta potential, and Ag^+ dissolution rate. Bone et al. (2012) stated that, similarly to the abiotic factors, biotic interplays between aquatic communities can change the concentration, speciation, and toxicity of AgNPs. They designed four microcosms with different environmental matrices –water only, water + sediment, water + plants (*Potamogeton diversifolius* and *Egeria densa*), and water + plants + sediment; then the water of the microcosms was used to study the acute toxicity in *D. rerio* and *D. magna* after 24 h exposure to the AgNPs of the microcosms. Results showed that the presence of aquatic

plants can decrease the toxicity of AgNPs and Ag^+, due to the decrease of the water column concentrations, or alternation of the surface chemistry of AgNPs with organic matter discharge from plants.

The use of the radioisotope (^{110}Ag) to trace the behavior of AgNPs with three various sizes (20, 50, and 100 nm), and their uptake by *D. magna*, showed that the penetration of AgNPs is dependent on the particle size in the media as illustrated by Zhao & Wang (2012). These authors found that ingestion was the main uptake route, due to the allocation of more than 60% of AgNPs in the gut of *D. magna*. In addition, at low AgNPs concentrations (5 µg/L), the uptake rate was lower than that of $AgNO_3$, while at high concentrations (500 µg/L); AgNPs uptake was higher than its ionic counterpart. Croteau et al. (2011) indicated that silver can accumulate in the body of the freshwater gastropod *Lymnaea stagnalis*, by either aqueous or dietary exposure to both forms of silver (AgNPs or Ag^+), but the uptake rate was faster in Ag^+-exposed organisms. Even though, due to the effects of AgNPs on the digestion process after dietary exposure, gastropods could not grow normally. Determining the biokinetics of AgNPs in *D. magna* by radiotracer methodology showed that the uptake rate of AgNPs is concentration-dependent (Zhao & Wang, 2010): at lower concentrations (2, 10, 40 µg/L), the uptake rate of AgNPs was from 0.06 to 4.3 times lower than that for Ag^+, while at 160 and 500 µg AgNPs/L the uptake rate increased disproportionately. Such findings are similar to those related with the direct ingestion of these nanoparticles by daphnids. Zhao & Wang, (2010) also reported that the main elimination occurred via water excretion for both Ag forms, but the result of the efflux rate revealed the difficulty of AgNPs elimination in *D. magna*.

The exposure of *Daphnia magna* neonates to various AgNPs by Li et al. (2010) revealed the dose-dependent toxicity, and the addition of food was able to decline the AgNPs toxicity. The toxicity of AgNPs to various life stages (eleutheroembryos, larvae, and juveniles) of the rainbow trout revealed the higher sensitivity of earlier life stages to AgNPs (0.25 mg/L, LC_{50} for eleutheroembryos), (Johari et al. 2013). Moreover, a dose-dependent decrease of blood plasma chloride and potassium, and dose-dependent increase of cortisol and cholinesterase in the juveniles exposed to AgNPs were also reported.

Recent studies have clearly shown that Ag^+ can inhibit the transport of Na^+ in fish species. For instance, Schultz et al. (2012) reported that both non-dialyzed and dialyzed AgNPs (1.0 mg/L citrate-capped) and Ag^+ (10 µg/L) can inhibit Na^+ penetration in rainbow trout juveniles. However, the inhibition of Na^+ K^+-ATPase, with no impact on carbonic anhydrase activity, can be considered as AgNPs specific effects.

Exposure of adult zebra fish to AgNPs can cause an increase in the malondialdehyde (by-product of cellular lipid peroxidation (LPO)) and total glutathione in the liver, through inducing oxidative stress, in addition to the increase in the DNA damage (Choi et al., 2010). The chronic exposure (21 days) of male Medaka to AgNPs at very low concentrations (1 µg/L) can enhance the transcription of stress-induced biomarker genes (e.g. metallothionins (MT) and Glutathione S-transferases (GST)), and estrogenic genes (e.g. Chg-L and VTG1), while such inductions were reduced under higher AgNPs concentration (25 µg/L), (Pham, Yi, & Gu, 2012). Fin regeneration was reported to be slower in zebra fish exposed to low concentration (0.4 ppm) of AgNPs (10-20 nm). In addition, changing in p53 gene expression of young zebrafish were exposed to AgNPs caused induction of apoptosis (Yeo & Pak, 2008). AgNPs and Ag^+ have been proved to enhance LPO and DNA damage, respectively, in rainbow trout hepatocytes: AgNPs may generate extracellular reactive oxygen species (ROS), while Ag^+ can generate intracellular ROS (Massarsky et al., 2014). Gagné et al. (2012) exposed *Oncorhynchus mykiss* rainbow trout to AgNPs (20 nm) and $AgNO_3$ for 96 h at 15 °C. They concluded that Ag^+ implicates metals mobilization and oxidative stress, but AgNPs cause inflammation and protein denaturation. The exposure of the freshwater snail *Lymnaea luteola* 96 h to AgNPs (32.40 ± 2.60 nm) rendered an LC_{50} of 48.10 µg/L (Ali et al., 2014). Moreover, Ali et al. (2014) have reported the decrease of glutathione, glutathione-s-transferase, and glutathione peroxidase, and the increase of malondialdehyde and catalase in the digestive gland of *L. luteola*. AgNPs (125-1000 g/L) can cause the destruction of the egg membrane, anaerobic metabolism and oxidative stress in Medaka embryos (Wu & Zhou, 2012). Exposure of freshwater mussel *Elliptio complanata* to AgNPs (20 and 80 nm) and Ag^+ for 48 h at 15 °C revealed that all forms of Ag can cause an increase in the MT and LPO, while the adverse effect of 20 nm AgNPs was more similar to Ag^+, as stated by Gagné et al. (2013). The same authors concluded that Ag^+ release is not the only way to show the toxicity of AgNPs. Exposure of *Nereis diversicolor* to 0, 1, 5, 10, 25, and 50 µg Ag/g dry weight sediment nano (<100 nm)-, micro (2–3.5 µm)- and ionic ($AgNO_3$)-Ag showed the highest genotoxic effects to AgNPs-exposed worms, while for those exposed to Ag^+, the toxic effects was minor. However, in terms of Ag bioaccumulation, no significant difference was observed between the three forms of Ag, (Cong et al., 2011).

Despite the impact of $AgNO_3$ on the LPO in the plasma of fish, exposure to AgNPs (10 nm) at high concentrations decreased the process in the gills. As shown by Wu et al. (2010), homogeneously released AgNPs (25 nm, 100-1000 µg/L) during 70 days can inhibit the

growth of embryonic Medaka following a U-shaped dose-response pattern. Moreover, acute toxicity tests on adult fish indicated an LC_{50} of 1.03 mg/L, and also 100% mortality of fish in 2.0 mg/L. In addition, Wu et al. (2010) proposed that complex mechanisms are involved in the AgNPs toxicity due to morphological abnormalities and non-linear dose-response patterns of growth retardation in the fish exposed to AgNPs. Table 2.1 summarizes the results of the recent studies on the toxicity of AgNPs on the freshwater organisms.

Table 2.1. A summary of the recent studies on the toxicity of AgNPs on the fresh water organisms.

Organisms	Endpoint	AgNPs LC_{50}/EC_{50}	Coating or size	Experimental design	Ag^{+} LC_{50}/EC_{50}	Reference
Pseudomonas putida	Growth inhibition	88 mg/L	35 nm	30 min	0.44 mg/L	Dams et al. (2011)
Tetrahymena pyriformis	Growth inhibition	1.463 mg/L	9 nm	24 h		Shi et al. (2012)
Pseudokirchneriella subcapitata	Growth	32.4 µg/L	Paraffin coating 3–8nm	72 h	33.79 µg/L	Ribeiro et al. (2014)
Chlamydomonas reinhardtii	Growth	1049 nM	25nm	2 h	184 nM	Navarro et al. (2008)
Lymnaea luteola L.	Mortality	48.10 µg/L	32nm	96 h		Ali et al. (2014)
Daphnia magna	Immobilization	72 µg/L	Paraffin coating 3–8nm	48 h, with food	3.38 µg/L	Ribeiro et al. (2014)
	Immobilization	11.02 µg/L	Paraffin coating 3–8nm	48 h, without food	1.04 µg/L	
	Mortality	0.41 µg/L	BPEI-AgNPs 100 nm	48 h		Silva et al. (2014)
	Mortality	2.88 µg/L	Citrate-AgNPs 100 nm	48 h		
	Mortality	4.79 µg/L	PVP-AgNPs 100 nm	48 h		
	Mortality	3.41 µg/L	Polyethylene glycol–coated AgNPs 8 nm	48 h	1.06 µg/L	Newton et al. (2013)
	Mortality	3.16 µg/L	Arabic–coated AgNPs gum 18 nm	48 h		
	Mortality	14.81 µg/L	Polyvinylpyrrolidone-coated AgNPs 39 nm	48 h		

Table 2.1. (Continued)

Daphnia magna	Mortality	1.0 µg/L	Coffee coated	48 h	1.1 µg/L	Allen et al. (2010)
	Mortality	1.1 µg/L	Citrate coated	48 h		
	Mortality	16.7 µg/L	Sigma Aldrich AgNPs (SA)	48 h		
	Mortality	31.3 µg/L	Uncoated; SA coated	48 h		
	Mortality	176.4 µg/L	The addition of food with SA coated	48 h		
	Mortality	2 -126 µg/L	10-80 nm	48 h	1.2 µg/L	Kennedy et al. (2010)
Danio rerio	Mortality	0.487 - 47.89 ppm		24 h		Cunningham et al. (2013)
	Mortality	128.4 µg/L	paraffin coating 3–8nm		78.32 µg/L	Ribeiro et al. (2014)
	Mortality	1.18 µg/L	8 nm	96 h	0.07 µg/L	Massarsky et al. (2013)
Oncorhynchus mykiss	Mortality	0.25mg/L	16.6 nm	Eleutheroembryos		Johari et al. (2013)
	Mortality	0.71mg/L	16.6 nm	larvae		
	Mortality	2.16mg/L	16.6 nm	juveniles		
Cyprinus carpio	Mortality	0.43 ppm	18 nm	96 h	0.33 ppm	Hedayati et al. (2012)
Pimephales promelas	Mortality	2 -126 µg/L	10-80 nm	48 h	6.3 µg/L	Kennedy et al. (2010)
Oryzias latipes	Mortality	1.03 mg/L	28 nm	48 h		Wu et al. (2010)
	Mortality	34.6 µg/L	49.6 nm	96 h	36.5 µg/L	Chae et al. (2009)

2.4 Toxicity of AgNPs on marine communities

So far some evidences have proved toxic effects of silver nanoparticles on marine biological communities. Salinity may change the behavior of AgNPs in aquatic environment, maybe due to the higher ionic strength of the saltwater AgNPs can show distinct toxicity impacts to the reported for freshwater living organisms (Lapresta-Fernández, Fernández, & Blasco, 2012). Gambardella et al. (2015) observed that the size of AgNPs increase in just 1 hour in seawater and, in higher concentrations, AgNPs can aggregate to microsize particles in this media. As a result of changing AgNPs stability, salinity may affect AgNPs toxicity. The 96 h acute exposure of the rainbow trout to Ag^+ at different salinities (15, 20, 25, and 30) revealed that the toxicity of Ag^+ increased with the salinity rise (Ferguson & Fhogstrand, 1998). Also in this study, the LC_{50} at 25 psu was 401µg/L, while in lower salinities (15 and 20 psu) no mortality was reported at the same Ag^+ concentration. The authors suggested that such observations were probably due to the incomplete hypo-osmoregulatory ability of the rainbow trout. In another study (Macken, Byrne, & Thomas, 2012), it was observed that the toxicity of polyvinylpyrolidone (PVP)-coated AgNPs increased greatly by increasing the salinity, fact attributed to the combination of AgNPs surface properties and the saline media.

By the dissolution of Ag^+ in seawater (salinity 33 psu), AgNPs have been reported to be toxic to the marine macroalgae *Ulva lactuca* (Turner, Brice, & Brown, 2012). Contrariwise, based on chlorophyll-a fluorescence reduction and Ag accumulation, the exposure of the macroalgae to AgNPs did not reveal any phytotoxic impact up to 15 µg/L (Turner, Brice, & Brown, 2012), but Ag^+ was found to be toxic even at low concentrations (2.5 µg/L). On the other hand, the mortality rate, aggregation in gut region, apoptotic cells, and DNA damage, increased in *Artemia nauplii* in the presence of nano-molar concentration of AgNPs (Arulvasu et al., 2014), while the percentage of hatching decreased.

The bioavailability of AgNPs in seawater has been assessed by a few studies by measuring total Ag concentration in the animal's body. The study on the Ag toxicity to the Iceland scallop *Chlamys islandica* by Al-Sid-Cheikh et al. (2013) showed the fast accumulation of all forms of Ag, and also their rapid elimination with half-life ranging from 1.4 to 4.3 days, and from 17 to 50 days for slow elimination compartments. AgNPs toxicity to *Mytilus edulis* and *Crassostrea virginica* revealed that the aggregates can considerably increase the uptake and bioavailability of AgNPs to suspension filter-feeding bivalves (Ward & Kach, 2009). Moreover, regarding the Ag accumulation pattern in the tissues of rainbow

trout juveniles exposed to AgNPs at three different salinities, it followed this decreasing order: accumulation of Ag in the liver > kidneys = gills > white muscles (Salari Joo, Kalbassi, Yu, Lee, & Johari, 2013). Cellular biomarker responses in marine (salinity 25 psu) eastern oysters, *C. virginica*, exposed to AgNPs (0.02, 0.2, 2.0, and 20 µg/L), and to Ag^+ (0.2 and 20 µg/L) indicated that gill tissues were more sensitive to Ag^+, while hepatopancreas' tissues were very sensitive to AgNPs (McCarthy, Carroll, & Ringwood, 2013). So, hepatopancreas tissues in filter-feeding bivalves are the main target for the AgNPs adverse effects, such as the enhancement of the cellular stress biomarkers. The chronic exposure of sheepshead minnows, *Cyprinodon varieglarus*, to low levels of AgNPs (10 µg/L) at 15 psu salinity caused higher silver burdens than in the case of Ag^+-exposed fish (Griffitt et al., 2012). Khan et al. (2012) results illustrated that the bioavailability of Ag^+ is about twice than that of AgNPs in the estuarine snail *Peringia ulvae*. In addition, Li et al. (2012) reported that Ag^+ may be accumulated by *Littorina littorea* mainly from seawater (i.e. more than from ingestion of pre- or co-contaminated algal food, *Ulva lactuca*) and, AgNPs was seen to be less bioavailable to *L. littorina* than Ag^+ in seawater (salinity 32.5 psu) maybe due to the formation of chlorocomplex.

In addition to the factors influencing the properties of AgNPs in seawater, as salinity, the presence of several life-stages in many marine organisms can also affect the overall toxicity of these NPs. Ringwood et al. (2010) observed the toxic effects of AgNPs on the embryonic development at the same concentration (1.6 µg/L Ag) which induced the lysosomal destabilization in the eastern oyster *C. virginica adults.*

However, García-Alonso et al. (2014) exposed eggs, larvae, juveniles, and adults of *Platynereis dumerilii* to various concentrations (0.1, 1, 10, 100 µg/L) and showed that early life stages are more sensitive to AgNPs than the older ones. Gambardella et al. (2013) exposed the sea urchin, *Paracentrotus lividus,* sperm to AgNPs (0.0001 to 1 mg/L) and found that this animal is suitable for nanotoxicity tests. They reported no dose-dependent response of the sea urchin to AgNPs evidencing that other physical and biological parameters are involved in the AgNPs toxicity.

Kalbassi et al. (2011) showed that AgNPs are more toxic in freshwater than in saltwater species. However, another study on the sea urchin, *Paracentrotus lividussea* show that AgNPs can cause higher toxic effects than Ag^+ in seawater (salinity 39 psu), including developmental defects –delayed development, bodily asymmetry and shortened or irregular arm, and behavioural change– and impacts especially on the swimming patterns (Siller et al.,

2013). Also, the presence of AgNPs may inhibit calcite formation in the sea urchin exposed to AgNPs (Pitticharoenphun et al., 2012).

Some studies have also shown that the exposure at higher temperatures can increase the toxic effects of AgNPs: in the marine microalgae *Dunaliella tertiolecta*it decreases the total photosynthetic performance (Oukarroum, Polchtchikov, Perreault, & Popovic, 2012). Fabrega, Zhang, et al. (2011) also showed a significant decrease in the volume and biomass of biofilm by the biofilm exposure to 200 µg/L of AgNPs in seawater, at 29 psu. The investigation of the AgNPs toxicity in the marine diatom *Thalassiosira weissflogii* by Miao et al. (2009) showed that AgNPs aggregate forming particles larger than 0.22 µm in seawater. The concentration of the Ag^+ released from AgNPs was significantly reduced by diafiltration or thiol complexation. Gambardella et al. (2014) stated that the skeletal bio mineralization process in *Paracentrotus lividus* can be disrupted by the exposure of male gametes to AgNPs (0.0001 to 1 mg/L) in seawater at 37 psu. Table 2.2 summarizes the results of the recent studies on the toxicity of AgNPs on marine organisms.

Table 2.2. A summary of the recent studies on the toxicity of AgNPs on marine organisms.

Organisms	Endpoint	AgNPs LC50/EC50	Coating or size	Salinity	Ag^+ LC50/EC50	Ref.
Ceramium tenuicorne	Growth inhibition	26.7 µg/L	polyvinylpyrrolidone coated 7 nm	30 ppt 7 d	2312.2 µg/L	Macken et al. (2012)
Platynereis dumerilii	Mortality and abnormal development		citrate-AgNPs humic acid capped AgNPs	30 ppt		García-Alonso et al. (2014)
Peringia ulvae	Uptake and Elimination rate		16.5 nm	17 ppt		Khan et al. (2012)
Tisbe battagliai	Mortality	30.8 µg/L	polyvinylpyrrolidone coated 7 nm	34 ppt 24 h	167.3 µg/L	Macken et al. (2012)
Artemia nauplii	Mortality	10 nM	30–40 nm	33 ppt		Arulvasu et al. (2014)
Paracentrotus lividus	Abnormal development		5-35 nm	39 ppt		Siller et al. (2013)
Oncorhynchus mykiss	Mortality	2.08 Ppm	25.9 nm	0.4 ppt, 96 h		Kalbassi et al. (2011)
	Mortality	19.58 ppm	25.9 nm	6 ppt, 96 h		
	Mortality	41.79 ppm	25.9 nm	12 ppt, 96 h		

2.5 Conclusion

The behavior of AgNPs has been proved to differ under different environmental conditions, which is attributed to their especial physico-chemical characteristics such as size, specific surface area, and surface charge. As a consequence, it is not surprising that any changes in their behavior can alter their toxicity towards the living communities in the receiving environments. Results of recent studies point a non-dose dependent toxicity of AgNPs, which is supported by the idea that different behaviours of AgNPs can be expected depending on the environmental conditions, affecting AgNPs concentration and availability. Several environmental factors may have remarkable impacts on the toxicity of AgNPs in different media, such as the existing biota, light irradiation, pH, temperature, exposure duration, and salinity. Salinity is a key factor in the marine environment that has a high influence on AgNPs toxicity. However, the studies on the AgNPs toxicity in marine organisms are scarce.

Two different scenarios seem to be involved in the toxic effects of AgNPs on the aquatic biological communities. AgNPs can either release Ag^+ or induce toxic effects due to their particles' specific size characteristics. Thus, any factor that is able to change either the rate of Ag^+ release from AgNPs and/or the size of particles' aggregations can potentially turn them into more or less toxic materials. Also, the surface charge of AgNPs can be changed when entering seawater. This can lead to the formation of larger aggregates compared with those formed in freshwater. Ultimately, the rapid aggregation may result in AgNPs sedimentation, also threating benthic communities.

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Chapter 3

Toxic effects of silver nanoparticles and silver nitrate on *Nassarius reticulatus* veliger

3. Toxic effects of silver nanoparticles and silver nitrate on *Nassarius reticulatus* veliger

3.1 Abstract

Silver nanoparticles (AgNPs) have been used in many applications due to their interesting properties such as the high antibacterial activity. This fact led to an increased release of AgNPs in the receiving environments, especially the aquatic ecosystems. This study constitutes the first report on the toxicity of AgNPs in *Nassarius reticulatus* larvae, as a biological model for marine living systems. Recently hatched larvae were obtained by egg capsules cesarean under laboratory conditions and exposed to the nominal concentrations (0.1, 1, 10, 100 µg Ag/L) of AgNPs and Ag^{+}, either in the presence or absent of the food, for up to 96 h. Larval mortality and swimming behavior (namely by monitoring larvae activity and velar arrests), were determined for each of the AgNPs concentrations tested from 24 to 96 h of exposure. AgNPs were more toxic than the Ag^{+}, based on the median lethal concentration (LC50) calculated. Moreover, the presence of food was proved to be an effective factor, causing a significant drop in the mortality of the *N. reticulatus* veligers exposed to AgNPs. Results also revealed that AgNPs (EC50-96 h 0.044 µg Ag/L) are more toxic to *N. reticulatus* veligers, impacting their swimming ability, when compared to Ag^{+} (EC50-96 h 1.044 µg Ag/L). Curiously, despite the negative effect of AgNPs detected in veligers swimming behavior, velum arrests were significantly decreased after exposure to Ag^{+} and no significant effect was detected in this endpoint after during the exposure to AgNPs.

Keyword: *Nassarius reticulatus*, Silver nanoparticles, Silver nitrate, Seawater, Velum arrest, Larval swimming.

3.2 Introduction

Silver nanoparticles (AgNPs) have been used in a wide range of applications such as textile manufacturing, personal-care productions, purification filters, cleaning and cosmetics products, food and beverage packaging, goods for children, laundry and clothing care, home furnishings, computer hardware, sporting goods, etc. (see Woodrow Wilson International Center for Scholars 2007). As a consequence, health and environmental concerns related to the discharge of AgNPs have been

rising in the recent years. Investigating the release of Ag from socks during washing, Geranio et al. (2009) reported that the amount of Ag released from the studied textile (0.3-377 µg Ag/g textile) is highly dependent on the techniques under which AgNPs are included in the textile structure, the synergistic effects of the chemical agents, and the mechanical stresses forced to the textile under the washing process. In this regard, the discharge of AgNPs into the aquatic environment would be very probable. Accordingly, it was already shown that, in the Eastern and Southern Europe, the concentration of AgNPs can reach values even greater than 0.3 ng/L in 10% of most exposed rivers, especially in July (Dumont et al., 2015).

The mechanisms and the severity of the toxic effects caused or induced to the aquatic biological communities are directly related to the properties of AgNPs, and their interactions with the caring environment. In the low ionic strength of freshwater ecosystems, AgNPs can stay suspended enough to be released into the marine ecosystems (Salari Joo, Kalbassi, Yu, Lee, & Johari, 2013). Under saltwater higher salinities, AgNPs reactions can enhance their toxic properties mainly by altering their surface characteristics (Moore, 2006) such as increasing surface charge (García-Alonso et al., 2014). Accordingly, Macken et al. (2012) reported that the toxicity of polyvinylpyrolidone (PVP) coated AgNPs on *Ceramium tenuicorne* is greatly enhanced by increasing salinity. Additionally, AgNPs sedimentation may increase when they form large aggregates when penetrating into the seawater (Stuart, Rees, Cullen, & Compton, 2013), increasing their toxicity to marine benthic communities, and even threating all marine life.

There are some evidence on the sensitivity of early life stages of freshwater organisms to AgNPs (see e.g., Johari et al., 2013). However, such studies on the marine organisms are rare. The annelid *Platynereis dumeriliito* (see García-Alonso et al. 2014) is one of the marine organisms studied, and the effects of AgNPs on its different life stages have shown the higher sensitivity of early stages, when compared with other age groups.

Nassarius reticulatus (Linnaeus, 1758) is a marine gastropod, ubiquitously distributed in coastal areas of the North Atlantic. It is a scavenger (carrion feeder), and is a well-known bio-indicator of tributyltin (TBT) pollution (see e.g., Barroso et al. 2011). This marine snail inhabits water depths of 0 to 20 m, being part of many types of benthic communities (Nehring, 2005). It also has a wide geographical distribution throughout European coastal waters, the Mediterranean, and the Black Sea (Fretter & Graham, 1994). The species has a strong dispersive potential, mainly attributed to the planktonic phase by a free swimming veliger larvae that is present in the initial stage of its life cycle (Stroben, Oehlmann, & Fioroni, 1992).

Because of the easy breeding of the adults in aquaria (Zupo & Patti, 2009), and also the possibility of collecting their capsules in the environment (see Barroso & Moreira 1998), the larvae of this species can be considered as an available choice for marine toxicity studies. However, the toxicity of AgNPs to marine gastropod larvae has not been carried out so far. So, our aim is to study the toxicity of AgNPs to *Nassarius reticulatus* larvae, and to compare the toxic effects of silver nitrate (ionic form) on this marine species.

3.3 Material and methods

3.3.1 Test organisms

Nassarius reticulatus adults were collected in Ria de Aveiro, (NW Portugal at 40° 38' 33.24"N | 8° 44' 06.69''W) in March 2014 during low spring tide. Fifty specimens were transported to the laboratory and kept in an aquarium with 15 L of artificial seawater, at 35 psu (prepared with Carbon–filtered tap water and artificial sea salt), aerated, at 18 °C and under 12:12 photoperiod. This broodstock was fed weekly on mussels collected at the sampling site and kept frozen until use, and the water was renewed after each feeding period. Adults spawned after one week, releasing egg capsules. Each egg capsule was carefully separated from the glass of the aquarium by using a scalpel, being then transferred to a 500 ml glass beaker with artificial seawater gently aerated. Egg capsules were monitored daily under a stereo microscope and the nearly hatching ones (Fig. 3.1) were identified as follows. Usually larvae hatch after 12 (±3) days of capsules release (Zupo & Patti, 2009). Each individual capsule normally contains about 100-120 larvae that, near hatching, present a clear eye-spot and a ciliated velum allowing them an active movement even before its release into the water column (Zupo & Patti, 2009). Once identified, nearly hatching larvae were released from egg capsules by cesarean using a needle under a stereo microscope. Larvae were then picked up by micropipette and transferred into each treatment beaker.

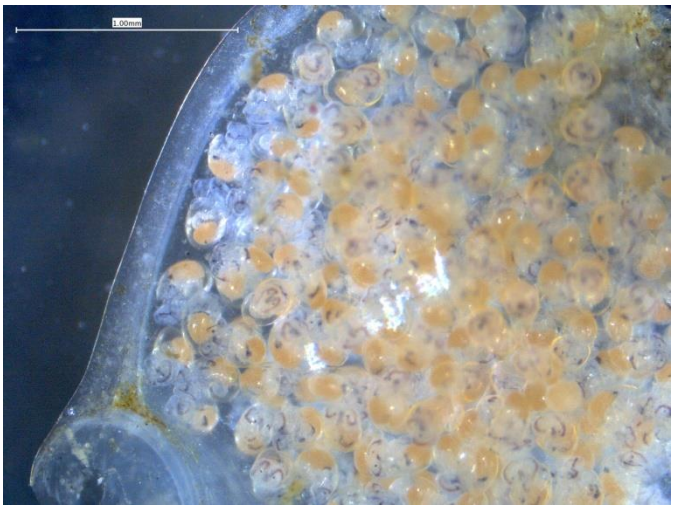

Figure 3.1. An egg capsule containing the nearly hatching *Nassarius reticulatus* larvae.Scale bar: 1 mm.

3.3.2 Chemicals

Silver nitrate purchased from Sigma-Aldrich with 99% purity and AgNPs (AMEPOX, 3-8 nm) were used in the toxicity tests. AgNPs with alkane coating were dispersed in water 1 mg/mL (Fig. 3.2). Stock solutions of Ag NPs and $AgNO_3$ were made using deionized water to prepare the required concentrations for acute toxicity experiments. Test solutions were also prepared through dilution of stock solutions in artificial seawater at 35-37 psu to achieve a salinity of 35 psu in all concentrations.

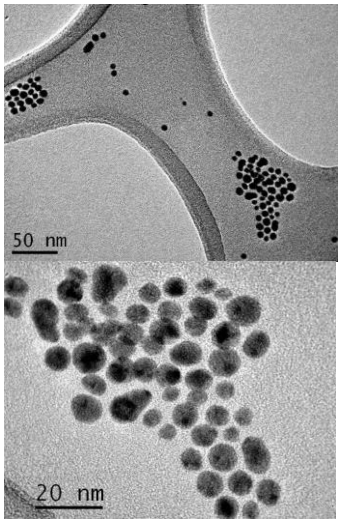

Figure 3.2. Transmission electron microscopy (TEM) images of the Ag NPs suspended in pure water.

3.3.3 Toxicity tests

In order to carry out the toxicity tests, artificial seawater was spiked with AgNPs and $AgNO_3$ to reach the different final concentrations of 0.1, 1, 10, and 100 µg Ag/L. A 24-well microplate was filled with 2.5 mL of Ag-spiked seawater, with 5 replicates per treatment. Unspiked seawater was used as control. Five newly hatched larvae were placed in each well. The test solutions were not renewed during the experiments. Tests were conducted either in the presence or absence of food, since suspended food particles may affect nanoparticles behavior (Ribeiro et al., 2014). The larvae were fed on the algae *Raphidocelis subcapitata* at a cell density of 2×10^4 algal cells/mL. All the experiments were performed according to two different procedures; with larvae from one single capsule, and with a mix of larvae obtained from four different egg capsules. In all the experiments, the larvae were monitored every 24 h up to 96 h under a stereo microscope. The following parameters were assessed: survival, swimming behavior and velar arrests. These parameters were established as our endpoints. The larvae which were swimming, or those which stayed at the well bottom but with the velum beating were counted as being alive (see Sousa et al. 2005). The larvae actively swimming in the solution were considered as being swimming in contrast to larvae that were lying at the bottom of the microplate well, without any response or active swimming after shaking the microplate. The arrest of each swimming veliger velum was counted during a period of 15 seconds to find out the effect of the presence of the AgNPs and $AgNO_3$ particles on each veliger velum beating (see, Braubach et al. 2006).

3.3.4 Statistical analysis

Normality test according to the Ryan-Joiner (similar to Shapiro-Wilk) procedure by using Minitab 17 software indicated the normality of the obtained data. Afterwards, the data were statistically tested by using one-way analysis of variance (ANOVA), followed by the Dunnett's post-hoc test at a P-value of 0.05. The LC_{50} (the concentration that caused 50% mortality) and EC_{50} (the concentration that caused a 50% decrease in the larval swimming) were determined by a non-linear regression with Minitab 17 software.

3.4 Results

3.4.1 Mortality

In the treatments with food, mortality in control ranged from 4 to 14% after 96 h. However, in the absence of food, mortality was higher than 20% in most control treatments after 96 h (Fig. 3.3). When the larvae from a mixture of four capsules (M) were exposed to AgNPs (0.0, 0.1, 1, 10, 100 µg Ag/L) in the presence of food, no significant difference in larval mortality in all treatments, compared with control, was observed in the first 48 h. After 72 h, the mortality of larvae significantly increased only at 1 µg Ag/L of AgNPs, and at 1, 10, 100 µg Ag/L after 96 h (Fig. 3.3 a). In the case of Ag^+, a significant larval mortality was found at 0.1 and 1 µg Ag/L after 24 h and at 0.1 µg Ag/L after 48 h, compared with control. But, after 96 h, mortality was significantly higher at all concentrations. In the presence of food, but with the larvae from just one single capsule (S), mortality was considerably higher in larvae exposed to AgNPs at 1 and 10 µg Ag/L than that in the control, after 72 h and 96 h (Fig. 3.3 a and c).

In the absent of food, when the larvae from four capsules were tested for AgNPs toxicity, a remarkable increase in the mortality was identified after 96 h in all concentrations, compare with the control (Fig. 3.3 a). For larvae from one single capsule, significant mortalities were found at 0.1, 1, 100 µg Ag/L after 72 h of exposure (Fig. 3.3 c). For the larvae under the same conditions, Ag^+ caused the higher mortalities than that of AgNPs at 100 µg Ag/L. However, for other concentrations, the mortality caused by Ag^+ exposure was lower than those exposed to AgNPs after 72 h (Fig. 3.3). LC50 values obtained are shown in Table 3.1. The standard deviation of mortality data of the veligers, exposed to AgNps and Ag^+ tests data are shown in Table 3.2 and 3.3, respectively.

Table 3.1. LC50 values calculated for *Nassarius reticulatus* veligers exposed to AgNPs and $AgNO_3$, either in the presence or absence of food in the exposure media. Larvae were collected from multiple capsules (n=4) and one capsule (n=1).

Test duration		LC50-72 h (µg Ag/L)		LC50-96 h(µg Ag/L)	
Particle		AgNPs	Ag^+	AgNPs	Ag^+
Presence of food	Multiple capsules	>100	>100	0.13	0.07
	One capsule	>100	>100	0.78	>100
Absence of food	Multiple capsules	0.32	10.72	0.05	*
	One capsule	1.60	66.15	*	43.96

*****LC50 was not possible to calculate.

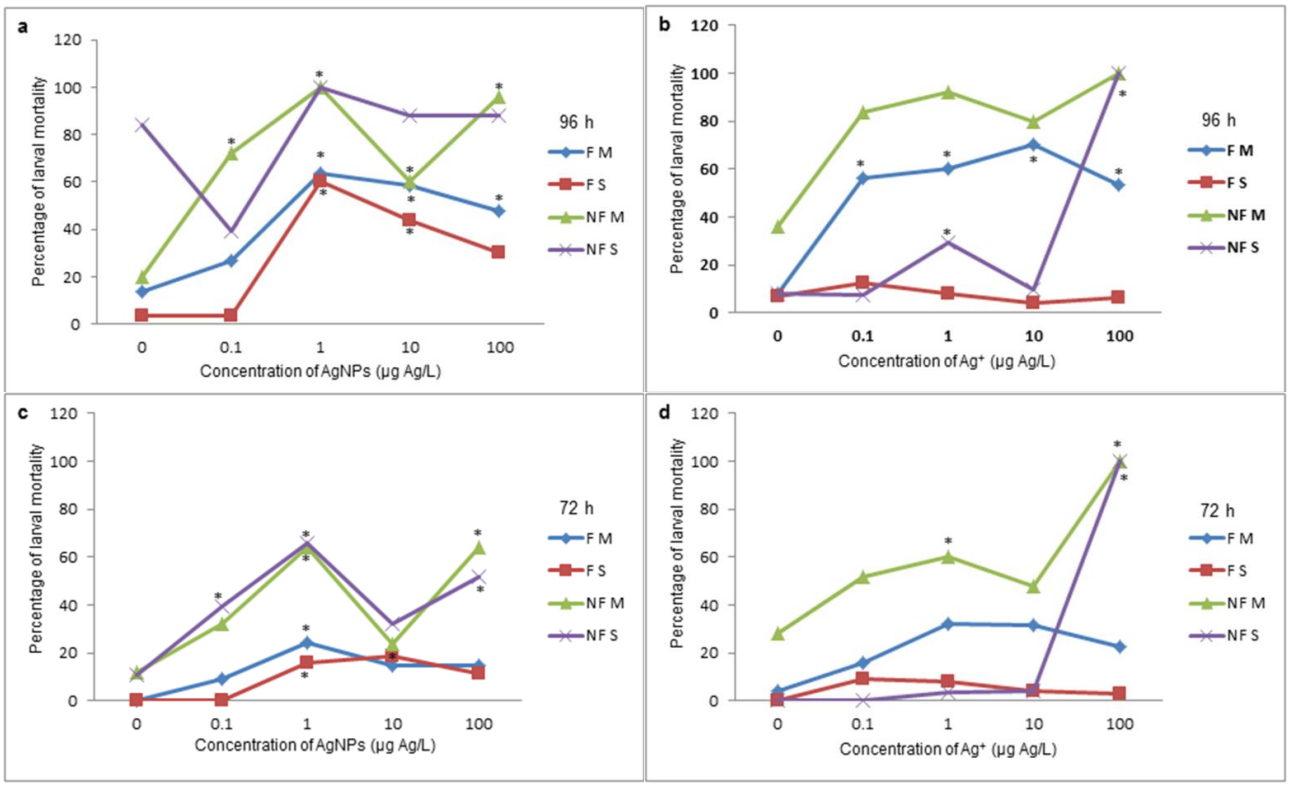

Figure 3.3. Mortality (%) of larvae exposed to a range of concentrations (0.0, 0.1, 1, 10, 100 µg Ag/L) of AgNPs (a and c) and Ag^{+} (b and d), in the presence (F) and absence (NF) of food. Experiments were carried out with larvae obtained from one capsule (S) and from a mix of four capsules (M). P <0.05 was considered as statistically difference, using Dunnett's method.

Table 3.2. Standard deviation and mean of the data related to the mortality of the *Nassarius reticulatus* veligers exposed to AgNPs.

Test duration	96 h								72 h							
Ag NPs	F M		F S		NF M		NF S		F M		F S		NF M		NF S	
Concentration	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev
0.0	13.71	19.21	3.33	7.45	20.00	20.00	84.0	26.1	0.00	0.00	0.00	0.00	12.00	10.95	10.67	15.35
0.1	26.76	22.15	3.33	7.45	72.0	30.3	39.43	19.31	9.05	13.08	0.00	0.00	32.00	17.89	39.43	19.31
1.0	63.90	22.15	60.0	40.0	100.00	0.00	100.00	0.00	24.38	10.00	16.00	16.73	64.00	21.91	66.00	13.42
10.0	58.67	21.42	44.00	5.48	60.0	28.3	88.00	10.95	14.67	14.45	18.667	1.826	24.00	16.73	32.00	17.89
100.0	48.00	8.37	30.00	14.14	96.00	8.94	88.00	17.89	14.67	16.43	11.33	10.43	64.0	32.9	52.00	16.43

Table 3.3. Standard deviation and mean of the data related to the mortality of the *Nassarius reticulatus* veligers exposed to Ag^{+}

Test duration	96 h								72 h							
Ag^{+}	F M		F S		NF M		NF S		F M		F S		NF M		NF S	
Concentration	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev
0.0	8.00	10.95	6.67	9.13	36.00	8.94	8.00	10.95	4.00	8.94	0.00	0.00	28.00	10.95	0.00	0.00
0.1	56.0	26.1	12.38	13.72	84.00	8.94	7.33	10.11	16.00	8.94	9.05	8.32	52.0	22.8	0.00	0.00
1.0	60.0	28.3	8.00	10.95	92.00	10.95	29.52	13.11	32.00	17.89	8.00	10.95	60.00	14.14	3.33	7.45
10.0	70.0	24.5	4.00	8.94	80.0	28.3	9.83	9.36	31.3	30.7	4.00	8.94	48.0	30.3	4.00	8.94
100.0	53.33	18.86	6.19	8.52	100.00	0.00	100.00	0.00	22.7	25.2	2.86	6.39	100.00	0.00	100.00	0.00

3.4.2 Swimming test

Swimming behavior in control animals showed great variation between treatments. Animals obtained from a mix of egg capsules presented lower percentage of active swimming than larvae from a single capsule, independently of the feeding regime (presence/absence) during exposure. AgNPs did not cause any significant effects on the percentage of the active swimming larvae originated from 4 capsules after 24, 48, 72 and 96 h. When larvae were exposed to Ag^{+}, the percentage of swimming larvae significantly increased at 100 µg Ag/L, compared to control, after 24 h of exposure, but after 48 h of exposure no significant effects were observed. The percentage of the actively swimming larvae from one capsule when exposed to AgNPs did not change from control after 24 h (Fig. 3.4a); however it significantly dropped dose-dependently after 48, 72 and 96 h of exposure. Moreover, it was also observed a decline in the swimming activity of the larvae originated from one capsule when exposed to Ag^{+}, remarkably at 0.1 µg Ag/L after 48 h, at 0.1, 1, 100 µg Ag/L after 72 h and at 100 µg Ag/L after 96 h (Fig. 3.4b).

In the absence of food, the impact of the AgNPs on the swimming activity of the larvae increased in the case of larvae originated from 4 capsules after 24 h at 100 µg Ag/L, and after 48 h at 10 µg Ag/L. While in the same conditions, Ag^{+} showed no significant toxic effects on larval swimming (Fig. 3.4 b). However, for the larvae from one capsule, the swimming activity decreased after 24 h of exposure to AgNPs in all concentrations and notably at 1 and 100 µg Ag/L (Fig. 3.4 a), while for the larvae exposed to Ag^{+}, such a decline was just observed in 100 µg Ag/L after 24 h (Fig. 3.4 b).

About the effects of the food presence on the AgNPs toxicity on larvae swimming, lower number of swimming larvae was observed in the non-fed treatments, compared with the fed ones in the control groups after 48. So, only 24 h duration was considered when studying the effect of the presence of the food on the larvae swimming, in order to eliminate the effect of the larvae harvest on their swimming behavior. In this regard, after 24 h, a significant decrease in the larvae swimming was observed in lower concentrations of 0.1 and 1 µg Ag/L after 24 h in absence of food for the larvae from 4 capsules. Such a decline was occurred for the larvae from one capsule in 1 and 100 µg Ag/L concentrations of AgNPs. However, Ag+ showed such adverse effects only in highest concentration (100 µg Ag/L) in the case of the food absence condition after 24 h for larvae from 4 capsules and also from one capsule.

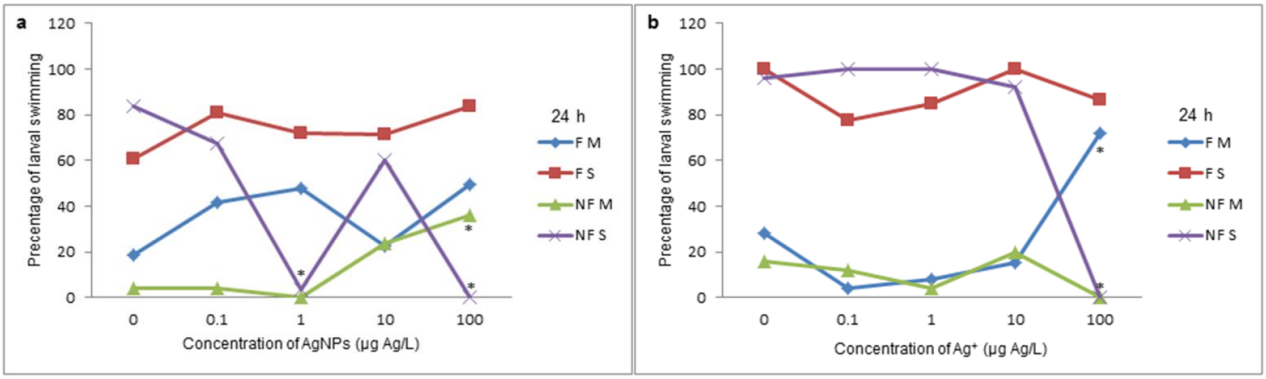

Figure 3.4. Percentage of larvae actively swimming during the exposure to a range of concentrations (0.0, 0.1, 1, 10, 100 µg Ag/L) of AgNPs (a) and Ag^+ (b), in the presence (F) and the absence (NF) of food. Exposures were carried out using larvae obtained from one capsule (S) and from a mix of four capsules (M). The P <0.05 was considered as statistically difference using Dunnett's method.

3.4.3 Velum arrest

Velum beating in control larvae varied from 0.75 to 3 units in the presence of food, and from 0 to 0.9 in the absence of food. AgNPs and Ag^+ did not affect the velar arrest average of the larvae obtained from a mix of 4 capsules after 24 h (Fig. 3.5a and 3.5b). Also, the average of velum arrests of the larvae obtained from one capsule was not affected by AgNPs after 24 h. For larvae exposed to Ag^+ under the same conditions, arrests of larvae velum significantly decreased dose-dependently after 24 h test duration compare with control (Fig. 3.5 b). In the absence of food, velum arrests of larvae extracted from both 4 and 1 capsule were not affected by AgNPs and Ag^+ (Fig. 3.5 a and 3.5 b). EC50 values can be found in Table 3.4.

The information related to standard deviation of the data related to the swimming and the velum arrest of the veligers exposed to AgNps and Ag^+ tests data are presented in Table 3.5 and 3.6, respectively.

Table 3.4. EC50 values of different endpoints in *Nassarius reticulatus* veligers exposed to the AgNPs and $AgNO_3$, either in the presence or absence of food in the media.

Endpoint	Treatment	EC50 AgNPs (µg Ag/L)	EC50 Ag^+ (µg Ag/L)	Duration
Swimming	With food one capsule (F S)	0.044	1.044	96 h
	No food one capsule (NF S)	1.08	30.18	24 h
Velar arrest	With food one capsule (F S)	>100	0.03	24 h

Table 3.5. Standard deviation and mean of the data related to the swimming of the *Nassarius reticulatus* veligers exposed to AgNPs and Ag^{+}.

Particle type	Ag NPs								Ag^{+}							
Swimming 24h	F M		F S		NF M		NF S		F M		F S		NF M		NF S	
Concentration	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev
0.0	18.86	14.37	60.7	37.9	4.00	8.94	84.0	26.1	28.0	30.3	100.00	0.0	16.00	8.94	96.00	8.94
0.1	41.9	32.8	80.67	14.22	4.00	8.94	67.4	23.1	4.00	8.94	77.4	23.4	12.00	17.89	100.00	0.0
1.0	48.1	23.0	72.00	10.95	0.00	0.00	3.33	7.45	8.00	10.95	84.67	8.69	4.00	8.94	100.00	0.0
10.0	22.67	22.29	71.33	19.38	24.00	21.91	60.0	28.3	15.33	16.60	100.00	0.0	20.0	28.3	92.00	17.89
100.0	49.33	18.77	84.0	26.1	36.0	26.1	0.00	0.00	72.0	39.0	86.29	19.21	0.0	0.0	0.0	0.0

Table 3.6. Standard deviation and mean of the data related to the velum arrest of the *Nassarius reticulatus* veligers exposed to AgNPs and Ag^{+}.

Particle type	Ag NPs								Ag+							
Velum arrest	F M		F S		NF M		NF S		F M		F S		NF M		NF S	
Concentration	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev
0.0	0.750	0.500	0.875	0.750	0.0	*	0.300	0.447	0.875	1.031	3.000	0.866	0.750	0.957	0.900	0.652
0.1	0.125	0.250	0.700	0.274	1.0000	*	0.300	0.447	0.0	*	0.900	0.548	0.750	1.061	0.500	0.354
1.0	1.100	1.475	0.300	0.447	*	*	0.000000	*	0.0	0.0	0.500	0.354	0.0	*	0.900	0.224
10.0	1.000	1.155	0.600	0.548	0.750	1.500	1.200	0.908	0.167	0.289	1.000	0.935	0.250	0.354	1.600	0.962
100.0	1.000	1.173	0.600	0.224	0.625	0.946	*	*	0.200	0.447	0.500	0.866	*	*	*	*

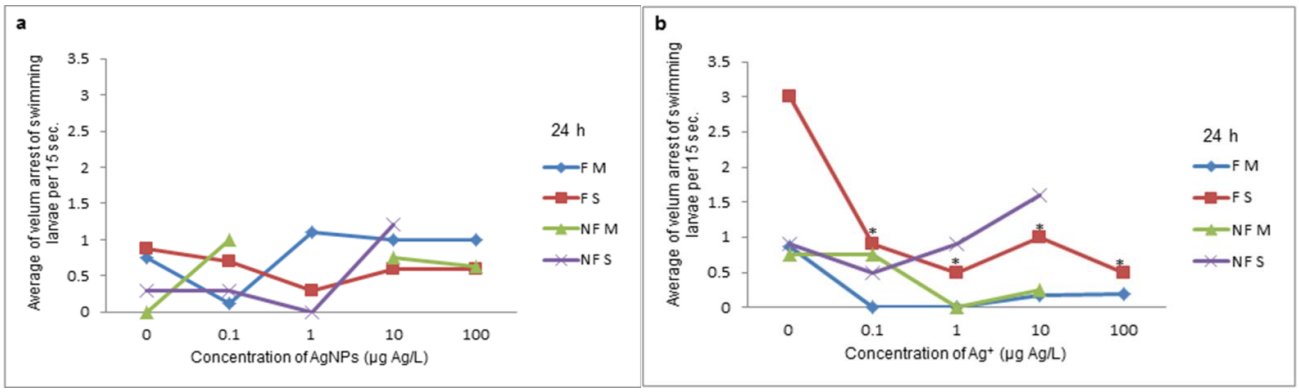

Figure 3.5. Average of velar arrests in swimming larvae exposed to a range of the concentrations (0.0, 0.1, 1, 10, 100 µg Ag/L) of AgNPs (a) and Ag^+, (b) in the presence (F) and the absent (NF) of food, carried out with larvae from one capsule (S) and mix of four capsules (M). The $P < 0.05$ was considered as statistically difference using Dunnett's method.

3.5 Discussion

Regarding the 96 h-LC50 of larvae originated from 4 capsules, Ag^+ (0.079 µg Ag/L) was more toxic than AgNPs (0.13 µg Ag/L). But, 96 h-LC50 of larvae extracted from one capsule showed such a different result (>100 µg Ag/L and 0.78 µg Ag/L for Ag^+ and AgNPs respectively) indicating the more sever toxic impacts of AgNPs on the larvae, in the presence of food. The variation in the LC50s related to the larvae extracted either from 1 or 4 capsules is probably related to the age and genetic properties of the veliger which can enforce their persistence when exposed to the Ag. Nevertheless, in the absence of food, AgNPs showed higher toxicity than Ag^+ in larvae from both one capsule and 4 capsules after 72h, with LC50 of 1.6 and 0.32 µg Ag/L for AgNPs and 66.16 and 10.72 µg Ag/L for $AgNO_3$. However, mortality in control larvae in the absence of food was high in some treatments, while mortality in control was below 20% in the presence of food. Thus, a combined effect of Ag and dietary (i.e. absence of food) cannot be ruled out.

The higher toxicity of AgNPs than Ag^+ on the marine organisms such as *Platynereis dumerilii* (García-Alonso et al., 2014), *Paracentrotus lividus* (Siller et al., 2013), *Tisbe battagliai* and *Ceramium tenuicorne* (Macken et al., 2012) at 30 psu (or more) has been also reported. However, Khan et al. (2012) revealed lower toxic effects of AgNPs on *Peringia ulvae* at 17 psu, compared to that caused by the Ag^+. Macken et al. (2012) showed the impacts of different salinities on AgNPs toxicity on *Tisba attagliai* and *Ceramium tenvicirne*. They reported an increasing trend in the larval mortality by increasing salinity. Also, Ferguson

& Fhogstrand (1998) indicated that the toxicity of Ag^+ on *Oncorhychus mykissin* increased with the salinity rise (15, 20, 25, and 30 psu). It should be highlighted that such results differ from the results obtained on some freshwater organisms such as *Chlamydomonas reinhardtii* (Navarro et al., 2008), *Daphnia magna* (Newton, Puppala, Kitchens, Colvin, & Klaine, 2013), *Danio rerio* (Massarsky et al., 2013), *Cyprinus carpio* (Hedayati, Shaluei, & Jahanbakhshi, 2012), *Oncorhynchus mykiss* (Gagné et al., 2012), *Pseudomonas putida* (Dams, Biswas, Olesiejuk, Fernandes, & Christofi, 2011). These studies clearly indicated lower toxic effects of AgNPs compared to those induced by Ag^+.

Based on the results of the present study, larval mortality did not increase in a dose-dependent manner when the larvae were exposed to AgNPs. The highest mortality occurred at 1 µg Ag/L, probably due to a higher agglomeration rate of AgNPs in higher concentrated test media. This result suggests that Ag bioavailability from AgNPs is dependent on the NPs characteristics in the media. McCarthy et al. (2013) showed that various types of interactions can be expected in different AgNPs concentrations, which may result in different toxic effects. So, it seems that the toxic effects of the AgNPs are independent on its concentration in the media. Other studies have also shown the dose-independency of the toxic effects of AgNPs to marine organisms. The results obtained by Gambardella et al. (2013) stated no dose-dependent inhibition of Acetylclolin esterase and Propinylecholinesterase in sea urchins exposed to AgNPs. Gambardella et al. (2015) revealed that, in seawater, the concentration of AgNPs is less important than other factors such as size and shape to determine the AgNPs toxic effects on *Paracentrotus lividus*. However, the toxicity of Ag may be affected by the duration of the exposure of marine organisms to Ag.

Our results clearly show a time-dependency for AgNPs toxicity on *N. reticulatus* larvae. Such observations are in agreement with other studies like those carried out on *Chlamydomonas reinhardtii* (Navarro et al. 2008), *Thalassiosira pseudonana* and *Cyanobacterium Synechococcus sp.* (Burchardt et al., 2012). The time dependency of AgNPs toxicity can be due to increasing release of Ag^+ from the surface of AgNPs by expanding the presence of the particles in the media. So, it is expected to release lower amounts of Ag^+ from the restricted surface of the agglomerated nano particles into the media (see Fig. 1.1). Moreover, the presence of food in the media can increase agglomeration of AgNPs, and, in its turn, can cause a significant drop in the mortality of the *N. reticulatus* larvae exposed to AgNPs in 0.1, 1, 100 µg Ag/L concentrations. However, only in the 100 µg Ag/L, Ag^+ was able to cause a significant higher mortality in the absence of the food. This process leads to an

increase of the sedimentation of the agglomerated Ag nanoparticles and, as a result, their bio-availability in the environment decreases. Ribeiro et al., (2014), by exposing three different organisms (the algae *Raphidocelis subcapitata*, the crustacean *Daphnia magna*, and the fish *Danio rerio*) to AgNPs and Ag^{+}, reported that toxicity of AgNPs is highly dependent on the presence of food, dissimilar to Ag^{+}. The decrease in the toxicity of AgNPs on *Daphnia magna* in the presence of food was also reported by Allen et al. (2010).

The swimming patterns of the larvae originated from 4 capsules in the control were quite variable. This is likely due to the effect of the growth stage or genetics properties of veliger on their swimming behaviours. The average number of actively swimming larvae in the control group was less than 30%; which decreased to 0% after 72 h in the presence of food; and after 48 h in the absence of food. However, the percentage of swimming larvae was relatively high in the control group, when the larvae were originated from one capsule. Consequently, it is suggested to choose the larvae from just one capsule when considering 'active swimming' as an endpoint for toxicity studies. The 96 h-EC50 of larval swimming in the presence of food (0.04 and 1.04 µg Ag/L for AgNPs and Ag^{+}, respectively), and also 24 h-EC50 in the absence of food (1.08 and 30.18 µg Ag/L for AgNPs and Ag^{+}, respectively) can support the idea that the toxic effects of AgNPs on the active swimming of larvae are more severe than that of Ag^{+}. The present study showed a dose-dependent decline in the larval swimming activity after 48 h of exposure to AgNPs in the presence of food. This finding is in agreement with the results achieved by Siller et al. (2013). They reported dose-dependent adverse impacts of AgNPs on swimming of sea urchin in the seawater (39 psu). Braubach et al. (2006) investigated the neural control of the velum in the larvae of the gastropod *Ilyanassa obsoleta*, and found that the serotonin increased the larva feeding and swimming at the top of a water column. Moreover, they observed that the catecholamines decreased larva feeding, and caused the concentration of the larvae at the bottom of the water column. Also, they showed that either 10^{-5}mol/L of serotonin or dopamine can significantly increase the average of the velum arrests. However, no clear effects of AgNPs on velum arrests could be identified in the present study. However, in the case of Ag^{+}, effects on velum arrests were dose-dependently decreased in the presence of food (EC_{50} of 0.03 µg Ag/L) which may be attributed to the ionic equilibrium disturbance.

The results of AgNPs toxicity test on *N. reticulatus* larvae in the present study, under which various endpoints (mortality, larval active swimming and velar arrest in swimming larvae) were considered, can suggest that different mechanisms are involved in the toxicity of

AgNPs and Ag^+ in marine invertebrate larvae and possibly in marine biological communities, as reported by Gagné et al. (2012) for the toxicity of AgNPs on *Oncorhynchus mykiss*. Moreover, it seems that *N. reticulatus* larvae might be a suitable model for AgNPs toxicity studies in seawater, due to their high sensitivity to very low concentrations of AgNPs (i.e., from 1 µg Ag/L). Other studies have shown that early life stages of marine organisms –e.g., *P. dumerlitto* (García-Alonso et al., 2014)– are more sensitive to AgNPs (Johari et al., 2013). However, Ringwood et al. (2010) stated that the toxic effects caused or induced by a certain concentration of AgNPs are the same on both embryos and adults of oysters.

In addition, AgNPs form higher-size agglomerates in seawater after a short period of time (1 h, Gambardella et al., 2015), which can cause rapid sedimentation of AgNPs in the marine environment. It must be stated that *N. reticulatus*, as a scavenger, is at high risk and threatened by the entrance of NPs such as AgNPs in the marine environment (Ward & Kach, 2009), mainly due to AgNPs precipitation in saltwater (Salari Joo et al., 2013). One of the reasons for this phenomenon is the potential reaction of AgNPs with compounds like Cl^- (Dai, 2012).

3.6 Conclusion

The present study considered several endpoints –mortality, larval active swimming and velar arrest of swimming larvae– in order to assess the toxic effects of AgNPs and Ag^+ on *N. reticulatus* veligers. Results revealed that AgNPs can induce more sever toxic effects than Ag^+ based on mortality and swimming behavior in this marine organism. The presence of food was identified as an effective factor on toxicity tests using this model since it can cause a significant decrease in the mortality of veligers exposed to AgNPs. The EC50-96 h of the swimming larvae exposed to AgNPs and Ag^+ revealed that AgNPs (0.044 µg Ag/L) is more toxic than Ag^+ (1.044 µg Ag/L) regarding the reduction of the larvae active swimming. On the other hand, velum arrests were significantly decreased in larvae exposed to Ag^+, while AgNPs did not affect the velar arrest. Finally, AgNPs toxicity seems to be different from Ag^+, due to the higher toxicity of AgNPs at lower concentrations (e.g., 1 µg Ag/L). Even though, the risk of the release of Ag from NPs into the aquatic environment is still unpredictable, especially in the seawater with different ionic strengths.

3.7 References

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Chapter 4

Main conclusions and final remarks

4. Main conclusions and final remarks

Nowadays AgNPs are used in several applications in different scientific and industrial fields. Hence, these particles release of into the aquatic environments seems to be unavoidable (Gottschalk, Sonderer, Scholz, & Nowack, 2009). However, AgNPs can present unpredictable behaviours under certain environmental conditions, mainly due to their specific characteristics (Levard, Hotze, Lowry, & Brown, 2012). Their high surface activity induces them to be absorbed / attached to other organic or inorganic particles, which can potentially alter their bioavailability (Liu, Jin, Cao, & Tang, 2014). On the other hand, salinity, as a main differential characteristic of seawater, can change AgNPs surface charge (García-Alonso et al., 2014). This process can itself lead to the formation of large agglomerated particles in the marine environment (Bian, Berninger, Fulton, & Brooks, 2013; Buffet et al., 2014; Gomes et al., 2013). The sedimentation of such aggregated particles can be considered as a big threat for benthic biological communities. However, there are no sufficient studies on the AgNPs toxicity to benthic organisms and its involving mechanisms.

The review of recent studies on AgNPs toxicity in aquatic organisms (chapter 2) enumerates various toxic effects of AgNPs toxicity on freshwater and seawater organisms. The impacts described in seawater may be related to this media higher ionic strength when compared with that of freshwater (Lapresta-Fernández, Fernández, & Blasco, 2012). It is believed that the biota (e.g., Bottero et al. 2011) and other environmental factors such as light irradiation (e.g., Shi et al. 2012), pH, temperature (e.g., Oukarroum et al., 2012), exposure duration, and salinity (e.g., Macken et al., 2012) have remarkable impacts on the toxicity of AgNPs to marine organisms. For instance, the presence of dissolved organic matter (DOM), high pH, and light irradiation can reduce the releasing rate of Ag^+, while sulphide or chloride can decline the AgNPs toxicity by binding Ag^+.

In the present study, *Nassarius reticulatus* larvae were exposed to AgNPs and Ag^+ (chapter 3). The results indicated higher toxicity of AgNPs, in terms of larval mortality and active swimming, compared with Ag^+. These results were obtained specially in larvae obtained from a single egg capsule, in which larvae should be exactly at the same life stage when compared with the larvae extracted from a mixture of 4 different capsules.

A non-dose dependent toxicity of AgNPs on mortality was registered, supporting the idea that the behavior of AgNPs can be altered by certain conditions, such as AgNPs concentration (McCarthy et al. 2013).

In this work, the toxic effects of AgNPs were estimated to be 23 times higher than that of Ag^+ on the swimming activity of the exposed veligers. Similar results were registered sea urchins by Siller et al. (2013). However, no significant effect was observed in the average of velum arrests in veligers exposed to AgNPs, while the exposure to Ag^+ induced a dose-dependent decrease of the velum arrests (EC50 of 0.03 µg Ag/L).

This work constitutes the first report of the toxic effects of AgNPs to marine gastropods early life stages, using *N. reticulatus* as a model. However, the risk of the release of AgNPs into the aquatic environments is still unpredictable, especially in chronic-exposures.

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