Universidade de Aveiro Departamento de Biologia
Ano 2016

Vasco Filipe de Castro Domingues

Toxicidade de nanopartículas de prata e nitrato de prata em planárias *Dugesia tigrina*

Toxicity of silver nanoparticles and silver nitrate to the freshwater planarian *Dugesia tigrina*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica do Doutora Susana Patrícia Mendes Loureiro, Investigadora auxiliar do Departamento de Biologia da Universidade de Aveiro e co-orientação do Doutor João Luís Teixeira Pestana, Investigador em pós-doutoramento do Departamento de Biologia da Universidade de Aveiro.

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Dedico este trabalho à minha mãe Olívia Maria Pereira de Castro Domingues e em memória do meu pai, Aires de Jesus Domingues.

o júri

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palavras-chave

nanopartículas de prata, nitrato de prata, toxicidade, regeneração, alimentação, locomoção, *Dugesia tigrina*.

resumo

Atualmente, as nanopartículas de prata (AgNP) são bastante utilizadas em vários produtos devido às suas propriedades únicas e excecionais, nomeadamente a sua potente atividade antibacteriana. Algumas das suas aplicações comuns são em têxteis, produtos cosméticos e tintas. Deste modo, é esperada a presença de AgNP nos sistemas aquáticos. Tendo isto em consideração, o objetivo deste trabalho é descrever os efeitos tóxicos de AgNP de diferentes tamanhos, e comparar estes efeitos com os induzidos pela exposição a $AgNO_3$ usando as características comportamentais das planárias da espécie *Dugesia tigrina*.

Foram efetuados testes de exposição aguda (96 h) e testes de exposição crónica (8 dias) onde foram avaliados parâmetros como a sobrevivência, a locomoção, alimentação e regeneração cefálica. Foram selecionadas AgNP de diferentes tamanhos (AgNP de 10-25 nm e AgNP 3-8 nm) e comparados os efeitos com os da exposição a $AgNO_3$, de modo a analisar se a toxicidade tem origem na libertação de iões Ag ou se é devida às propriedades das diferentes nanopartículas.

Os resultados mostraram que os valores de LC50 para as 24, 48, 72 e 96 h não variaram ao longo do tempo para AgNP (10-25 nm) (76.62 µg L^{-1}) e para $AgNO_3$ (109.1 µg L^{-1}). As planárias experienciaram nas primeiras 24 h várias alterações morfológicas na zona da cabeça como aurículas suprimidas e principalmente dissolução da cabeça. Estes efeitos notaram-se principalmente na AgNP (10-25 nm) e $AgNO_3$. Em relação à exposição crónica, as planárias apresentaram uma redução significativa na locomoção e na alimentação na exposição a todas as nanopartículas estudadas, sendo estes parâmetros os mais sensíveis para *D. tigrina*. Relativamente à capacidade de regeneração não houve efeitos significativos à exposição a Ag. A fonte de toxicidade pode estar relacionada com as propriedades das AgNP que interferem com o sistema nervoso das planárias, causando a sua morte.

Este estudo demonstrou que as planárias são um organismo adequado para estudos ecotoxicológicos comportamentais e devem ser considerados em metodologias de avaliação de risco ambiental.

keywords silver nanoparticles, silver nitrate, toxicity, regeneration, feeding, locomotion, *Dugesia tigrina*.

abstract

Currently, silver nanoparticles (AgNP) are widely used in several products because of their unique and exceptional properties, particularly its potent antibacterial activity. Thus, AgNP are very often applied in textiles, cosmetics and paints. Under those circumstances, AgNP is expected to be present in aquatic systems. Taking this into consideration, the objective of the present work is to describe the toxicity of AgNP of different sizes and compare to the toxicity from $AgNO_3$ exposure using behavioral endpoints of the planarian *Dugesia tigrina*.

Acute exposure tests (96 h) and chronic exposure tests (8 days) were performed, in which parameters such as survival, locomotion, feeding and regeneration were evaluated. Therefore, AgNP of different sizes were selected (AgNP of 10-25 nm and AgNP 3-8 nm) and effects from exposure were compared to those from $AgNO_3$, in order to analyze whether the source of toxicity was originated by release the ionic form of Ag or related to the inherent properties of nanoparticles.

The results showed that LC50 values at 24, 48, 72 and 96 h were equal over time for AgNP (10-25 nm) (76.62 µg L^{-1}) and for $AgNO_3$ (109.1 µg L^{-1}). In the first 24 h, planarians experienced several morphological alterations at the head region such as suppressed auricles and mainly head dissolution. These effects were noted mainly in AgNP (10-25 nm) and AgNO3 exposures. Regarding chronic exposure, planarians presented a significant reduction in locomotion and feeding activity upon both AgNP exposures. These endpoints revealed to be the most sensitive to *D. tigrina.* There were no significant effects on the regeneration test. The source of toxicity may be related to the properties of AgNP that interfere with nervous system of planarians consequently causing their death.

This study demonstrated that planarians are an adequate organism for behavioural ecotoxicological studies and should be considered in environmental risk assessment methodologies.

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Chapter 1

General Introduction

1. General Introduction

1.1. Nanoparticles: an important segment of Nanotechnology

Nanotechnology is an emergent field in industry and science and it has been growing at high pace in the recent years. The global market value for nanotechnology was estimated at 14 billion euro in 2010, it is currently estimated at 24 billion euro and higher values that can reach 70 billion euro are foreshadowed for 2020. In such a scenario, many industries in different areas have opportunities to explore this fast growing market and consequently nanotechnology will become part of modern everyday life (http://www.prnewswire.com/).

One of the largest segments of nanotechnology are nanomaterials, particularly nanoparticles. Nanoparticles research has become very popular due to their novel properties with potential applications in several areas such as medicine, electronics, energy and food & agriculture sectors (Li & Chen 2015; Tarascon et al. 2000; Raccichini et al. 2015; Nair et al. 2010). More recently, a new era of consumer products based on nanoparticulate metals comes out such as clothes, lotions, cosmetics, paints and food packaging (Mitrano et al. 2015). Subsequently, there is a potential hazardous effects of nanoparticles to human health and ecosystems. It is important to realize that nanoparticles already exist on Earth before any anthropogenic production. In fact, their presence occurs naturally on the planet for millions of years. As an example, iron particles and silicates of 16-27 nm were found in a Cretaceous-Tertiary boundary layer (66 million years old) and volcanos have been responsible for releasing nanoparticles into the environment (Verma & Upadhyay 2002; Lee & Richards 2004). At the present, tons of natural nanoparticles are produced globally, including atmospheric dust, soil colloids, inorganic minerals and components of phytoplankton found in waters (Sharma et al. 2015; Wigginton et al. 2007; Ernst 2012).

There is a need to take into account safety and toxicity of nanoparticles by performing a risk assessment. There are several articles of EU legislation and technical guidance that support the implementation of legislation containing specific references to nanomaterials. In 2011, the European Commission aimed to adopt a definition of nanomaterial considering several aspects such as origin of nanomaterial, size range,

particles thresholds, surface area by volume and internal and surface structures. According to this definition nanomaterial means a natural, incidental or manufactured material containing 50% or more of the particles being within the number size distribution of 1 -100 nm in at least one of the dimensions and with a specific surface area by volume greater than 60 m^2/cm^3 (EC, 2011a). The objective of the definition is to serve as a reference to identify nanomaterials and support the legislation and regulatory frameworks to help the development of policy, guidance and risk assessment methods applicable to nanomaterials (Liden 2011).

Nanoparticles have size dependant properties as opposed to larger matter which have constant physical properties. As a matter of fact, nanoparticles have higher surface to volume ratio and particle number per unit mass than larger particles for the same mass and chemical composition (Navarro et al. 2008). As an illustration, Figure 1 shows different size materials with similar mass: one microparticle with a diameter of 60 µm (about the size of human hair), 1 million particles 600 nm diameter and 1 billion nanoparticles 60 nm diameter.

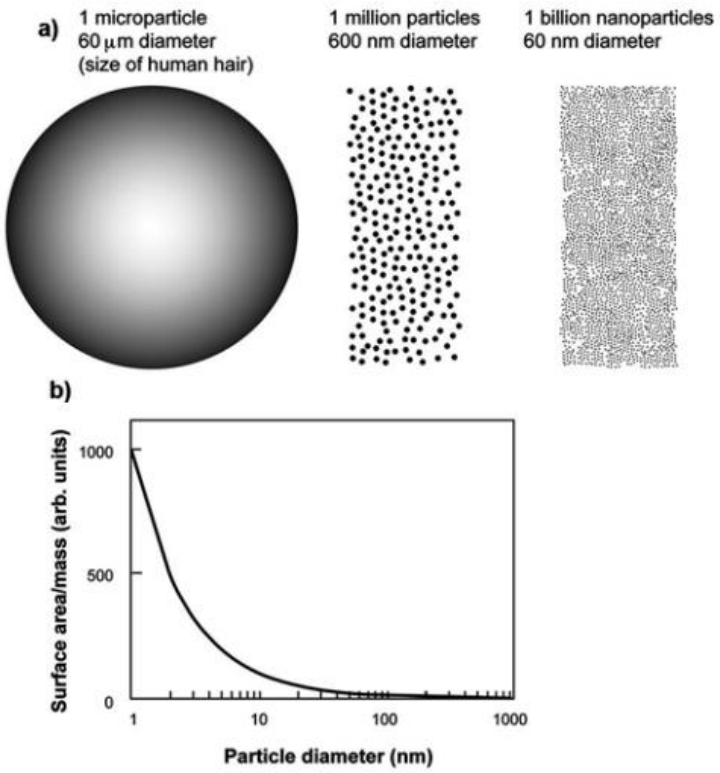

Figure 1. Scheme illustrating a) three different particles of similar mass and different individual size and b) a graph representing the relationship between particle diameter and surface area normalized by mass (Adapted from Buzea et al. (2007)).

Considering the same mass, particles with lower diameter have higher ratio of surface area to volume, therefore the ratio of surface area to volume particles of 60 nm volume is 1000 times higher than a particle with a diameter of 60 µm. For this reason, nanoparticles usually have an increased reactivity and distinctive physico-chemical properties when compared to the larger particles (Buzea et al. 2007).

1.2. The rise of silver nanoparticles

- *Silver*

Silver's chemical symbol (Ag) is derived from the Latin "argentum" which means "shiny" or "grey". Silver presence in the environment occurs naturally specially on Earth's crust as a minor constituent and it is found throughout the world. Historically, this metal has been extracted through mining activities and has been widely used in several applications such as coins, jewellery, utensils and photographic processing or development materials. Silver has also been used for water purification in swimming pools and home water treatment (Purcell & Peters 1998).

What makes silver appealing in a variety of applications is its exceptional properties. Silver is known to exhibit the highest electrical and thermal conductivity among all metals and the highest visual light reflection (about 97%). It is easily worked, possess high fatigue and corrosion resistance and is relatively nonreactive and nontoxic in its metallic form (Etris 2010).

- *Silver nanoparticles*

Silver nanoparticles (AgNPs) when compared to their larger counterpart possess unique properties with great industrial applications. According to the Consumer Products Inventory, a database that includes 1628 nanoparticles containing products, AgNP are the most widely used nanoparticle, present in 435 products (24%) (Vance et al. 2015). AgNP main properties are high thermal and electrical conductivity, high catalytic activity and chemical stability which make them valuable in several areas such as microelectronics and medical devices (Krutyakov et al. 2008). Furthermore, AgNP demonstrate a powerful

antimicrobial activity that makes them attractive in many applications, particularly in commercial daily used products like textiles, sun lotions, cosmetics, food packaging, biocide sprays and adhesives (Abbasi et al. 2014). In addition, there is an increasingly application of AgNP for antimicrobial coating in dressing wounds and biomedical devices (Rai et al. 2009).

Usually AgNPs are prepared through chemical route based on chemical reduction of silver nitrate, although they can also be synthesized using physical photochemical or biological methods (Tran et al. 2013). Each method has its own advantages and disadvantages regarding costs, scalability, particle sizes and size distribution and so far it is not clear whether different preparation methods affect AgNPs efficacy. During synthesis process, the size, shape and surface coating can be selected and controlled (Ali et al. 2014).

Due to their wide application in every day products there is a concern about the potential negative effects of AgNP. Nowadays, there are several studies and data aiming to help consumers, scientific organizations and policy makers by informing on exactly which products contain silver or other nanoparticles and addressing risk assessment with regards to environment and public health (McShan et al. 2014).

1.3. Presence of silver nanoparticles in the aquatic environment

Literature suggest that AgNPs enter into aquatic systems and their release is related to product life-cycle like production, transport, consume and disposal (Mitrano et al. 2015). Actually it is described that AgNPs can leach out from textiles and food packaging plastics (Benn & Westerhoff 2008). AgNPs are embedded into fibres or applied at material surface such as t-shirts, socks, underwear and many others materials entering the water system via washing effluent or through disposal (Blaser et al. 2008). Eventually, most of the AgNPs released will enter into sewer systems and thus reach wastewater treatment plants (WWTP). Once in WWTP, majority of Ag is retained in sewage sludge and a small amount is kept in the effluent stream (Kaegi 2011). When properly processed, sewage sludge can be transformed in biosolids which are used as fertilizer to improve soils performance and stimulate plant growth. Biosolids containing AgNPs will be applied

to agricultural soils and there is a possibility of leaching through soils entering in the aquatic systems (Brar et al. 2010).

Studies regarding the presence, mobility and fate of AgNPs in waters propose that several processes occur such as aggregation, precipitation, adsorption, sulfidation and dissolution consequently affecting its bioavailability and toxicity (Levard et al. 2012). Firstly, AgNPs might remain as individual particles in suspension and transported through water (Liu & Jiang 2015). Ag^+ oxidation to and Ag^+ dissolution from AgNPs are expected after contact with oxygen and other oxidants. Frequently, AgNPs flows in WWTP transformed in Ag_2S NP's after oxidation (Liu et al. 2011). Also, Ag^+ may react with chloride to form AgCl species (Levard et al. 2013). Dissolution of Ag^+ depends on dissolved oxygen, pH, temperature, salinity and AgNP size and concentration. Another important process that has been reported is aggregation which is also dependent on environmental factors such as pH, electrolyte composition and solution ionic strength. Aggregation of AgNP appears to strongly affect others processes like precipitation, dissolution and adsorption and thus particularly important in determining the fate of AgNP in aquatic systems (Luoma 2008). Generally speaking, the presence and fate of AgNP depends on these physico-chemical transformations which are influenced by properties of AgNPs and by the surrounding environment.

Lau et al. (2016) performed an aquatic microcosms experiment using different ionic strength waters and different coated AgNP (polyvinylpyrrolidone (PVP) and citrate) to study its transport and behaviour. Their results demonstrated that AgNP coated with PVP remain unaltered while AgNP coated with citrate were unstable. Also, higher electrolyte water favours aggregation which was the dominant process occurring followed by sedimentation. There was a decrease on Ag concentration due to its sorption to the wall of microcosms. AgNP may interact with chlorine and sulphur species increasing their persistence. To sum up, coating surface and water chemistry demonstrated a significant impact on AgNP stability. Seitz et al. (2015a) investigated the effect of media pH and presence of dissolved organic matter on the toxicity of various AgNP using *Daphnia magna*. It was concluded that higher pH and presence of dissolved organic matter as well as size and surface coating reduced toxicity to *D. magna*. In summary, it was observed that environment factors and NP's characteristics affects the toxicity of ion release from AgNP.

The Predicted environmental concentration (PEC) for total Ag and AgNPs for the freshwater environment is estimated at ng L^{-1} ranges (Mueller & Nowack 2008), however

considering a continuous increasing release of Ag, PEC can reach µg L^{-1} ranges, high enough to represent risk to aquatic organisms. Gottschalk et al. (2011) modeled PEC values for total Ag in surface waters calculating a PEC value of 0.76 ng L^{-1}. Also, Blaser et al. (2008) reported a PEC value of 40 ng L^{-1} in river water and 2 µg L^{-1} n sewage treatment plants. Due to vast production and diverse applications of AgNP it is expected to found nanoparticles differing in size, chemical composition, shape and coating material in aquatic environments (Mcgillicuddy et al. 2016). Unfortunately, no validation for these values have been carried out due to the incapability of the available techniques to overcome problems related to low concentrations or several confounding factors (e.g. presence of other particles in suspension) that may be found in real scenarios.

1.4. Ecotoxicity of silver nanoparticles

The toxicity of AgNPs has been reported in many ecotoxicological studies involving different group of organisms such as plants, fungi, algae, invertebrates and vertebrates (Gonçalves et al. 2016; Krishnaraj et al. 2016; Gubbins et al. 2011). Also, toxicity of AgNP has been also observed in microorganisms and even in human cells (Lu et al. 2010; Matzke et al. 2014).

Literature regarding the ecotoxicity of AgNP suggests that several sources within a NP structure are responsible for their toxicity. Most studies suggest that toxicity is due to the release of Ag^+ from AgNP to the environment eliciting a physiological response in organisms and alter their homeostasis. Others studies imply that Ag^+ release is not the only explanation, and some authors relate toxicity of AgNP to oxidative stress generated by the formation of reactive oxygen species (ROS), possibly formed at the surface of the AgNPs. Also the interaction between Ag^+ with groups of enzymes and proteins has ben reported, affecting cellular respiration and transport of ions across membranes, causing cell death (Sondi & Salopek-Sondi 2004; Farkas et al. 2010). In addition, toxicity of AgNP can be a combination of these mechanisms, being important to identify which part of toxicity is attributed to ionic form or to the nanoparticle form. Moreover, toxic effects of AgNPs can be linked to factors such as shape, chemical composition, interaction with biological biomolecules, aggregation changes and environmental transformations (Beer et al. 2012).

Figure 2 illustrates some processes related to fate and toxicity of AgNP occurring in environmental media. Once AgNPs reach the environment, several phenomena can occur, namely, displacement of the coating agents due to interaction with other molecules such as inorganic ions allowing aggregation and agglomeration; dissolution of AgNP into Ag^{+}; and interaction of silver atoms interaction with oxygen forming silver oxide (Ag^{0}). AgNPs can penetrate cells and become internalized acting as a source of Ag^{+} inside the cells. Once within the intracellular environments one of the main mechanisms of toxicity of AgNPs is oxidative stress through the generation of ROS causing damage to cellular components including DNA, depletion of antioxidant molecules such as glutathione, disabling proteins function and damage to the cell membrane (Misra et al. 2012; Kim & Ryu 2013; Ho et al. 2010).

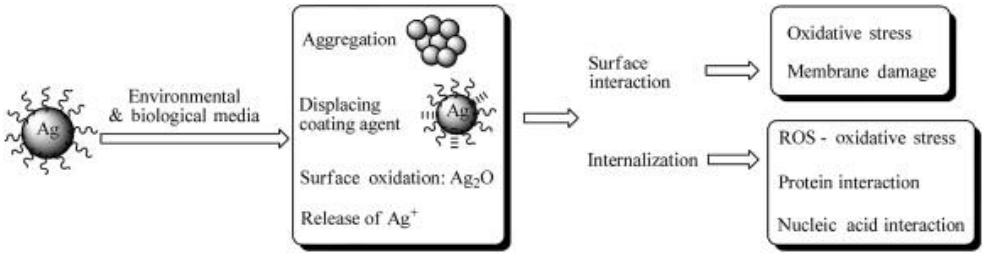

Figure 2 - Fate and toxicity of silver nanoparticles in environmental media. From McShan et al. (2014).

Usually AgNPs toxicity is studied comparing to $AgNO_3$ in order to observe if toxicity is related to ion release or properties of nanoparticles. Silver ions induce high toxicity to organisms mostly at µg L^{-1} ranges. $AgNO_3$ and AgNPs presents LC50 values around: 78.32 µg L^{-1} and 128.4 µg L^{-1} for zebrafish *Danio rerio*, respectively (Ribeiro et al. 2014); > 15 µg L^{-1} and 81.6 µg L^{-1} for snail *Physa acuta*, respectively (Gonçalves et al. 2016). On the other hand, for more sensitive organisms, such as algae *Raphidocelis subcapitata*, the EC50 values has been reported to be lower: 33.79 µg L^{-1} ($AgNO^{3}$) and 32.4 µg L^{-1} (AgNP) (Ribeiro et al. 2014); and 1.1 µg L^{-1} (Angel et al. 2013) and 26 nM (Lee et al. 2005) for $AgNO_3$. Also, *Daphnia magna* presents low EC50 values for $AgNO_3$ and AgNPs: 1.05 µg L^{-1} and 10.2 µg L^{-1}, respectively (Ribeiro et al. 2014); 1.10 µg L^{-1} and 121 µg L^{-1}, respectively (Völker et al. 2013). For the bacteria *Pseudomonas putida* it was observed $AgNO_3$ EC50 values of 0.16 µg L^{-1} (Matzke et al. 2014) and 10 µg L^{-1} for *Escherichia coli* (Ivask et al. 2014).

Most of ecotoxicological studies that evaluate the potential toxic effects of AgNPs in freshwater environment perform specific and standardized protocols for organisms and endpoints representing a restrict range of species. Bioavailability and effects of AgNP are often related to organism's life traits. With this in mind, non-standardized organisms, with different life traits will contribute additional and important information to AgNPs risk assessment.

1.5. The freshwater planarian *Dugesia tigrina*

- *Geographic range and habitat*

D. tigrina are benthic invertebrates originated from North America and unequivocally introduced into Europe due to activities of man. These animals are usually found in lakes, rivers and streams and, there are reports of dense populations of *Dugesia tigrina,* one of the most common species of flatworms, in Great Britain and Japan. *D. tigrina* is a benthic species showing negative phototaxis, thus tending to move away from light and for that reason are usually found under rocks, plant material or other type of debris (Ball & Fernando CH 1969).

- *Description*

This species presents a flattened dorsoventrally body covered with cilia on surface, ranging from 9 to 15 mm and are typically brown with yellow or white spots across the body. Figure 3 shows a diagram with the main structures of flatworms: head region exhibit photoreceptors, auricles and cephalization. These animals have two photoreceptors, the "eyes" of planarian, which sense light direction and intensity and respond to it by moving away from light (negative phototaxis). Planarians also have two auricles (the triangular extensions) functioning as sensory lobes. In addition, *D. tigrina* exhibits a particular feature: cephalization followed by nervous tissues (brain). When planarians receive light stimulation, photoreceptors convert light energy into signals that are transmitted through the neural receptors to the brain. Once received signals are sufficient, they are sent to the cilia which allows the planarian to move away from light. Sensory lobes also are linked to the brain allowing to recognize the presence of other organisms, detect food and respond to environmental stimuli like water movement (Krugelis MacRae 1964).

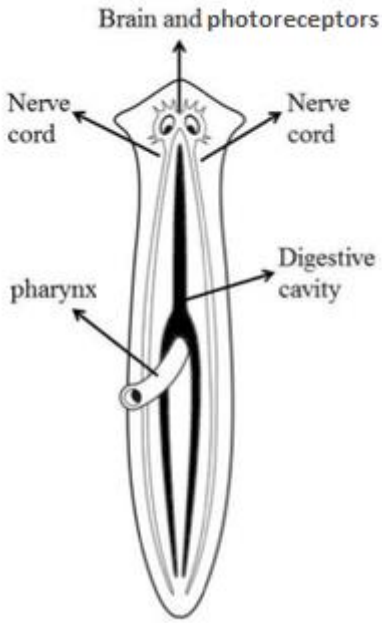

Figure 3. Diagrammatic representation of basic anatomy of planarian *Dugesia tigrina.* Adapted from The First Brain: The Neuroscience of Planarians.

- *Regeneration*

Planarians possess a remarkable biological characteristic: regeneration capability. They are able to fully regenerate when suffering damage to their tissue as well as restore missing parts from any small fragment of their body. Planarians can be cut into hundreds of pieces and each will develop into the whole planarian. Figure 4 shows a planarian transversely split in three parts and after regeneration process originated three planarians. This process is based on the proliferation of abundant populations of stem cells spread through planarian body, the neoblasts. After injury signal, neoblasts proliferate and form a mass of unpigmented cells called blastema at the site of wound. Over time, these cells will grow and differentiate, repairing and remodelling the missing body part. The region above the photoreceptors and the pharynx do not regenerate because of the nonexistence of neoblasts (Reddien & Alvarado 2004).

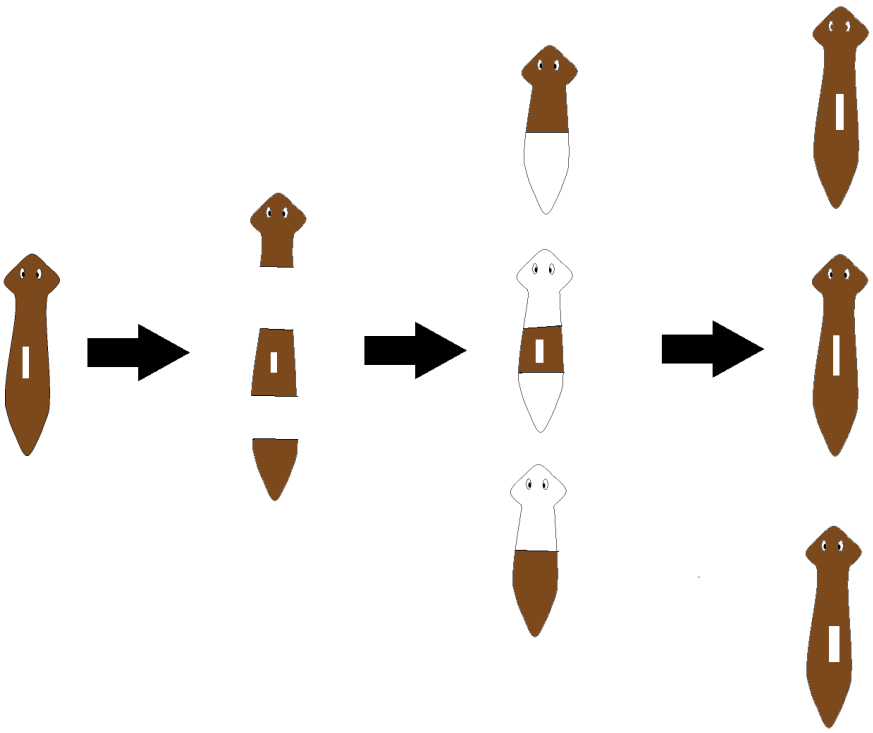

Figure 4. Scheme representing regenerative capacity of the freshwater planarian *Dugesia tigrina* after transverse cutting.

- *Food habits*

Regarding eating habits, *D. tigrina* is an opportunistic predator of small crustaceans, worms, larvae and insects. Sensory lobs of planarians detect the presence of other organisms and they can move, trap and capture their prey using mucus secretions, followed by the ingestion through the pharynx (Davies & Reynoldson 1971; Cash et al. 1993).

1.6. Planarians in ecotoxicology

Planarians present a set of biological characteristics that make them very relevant in ecotoxicology.

Planarians have a natural wide distribution in unpolluted streams, rivers and lakes. In a food web they represent an important level where they predate several organisms

(e.g. crustaceans, snails) but they can represent also a food source for fish. In the laboratory a large number of individuals can be achieved and maintain due to their regeneration capacity. They possess important behavioural characteristics that can be used as endpoints to evaluate toxicological effects such morphological changes like body depigmentation, head dissolution, twisted body, impairments in locomotion. Regeneration is a process of cell growth and it can be also included as an endpoint process, as it can be expected that some contaminants may induce apoptotic effects or inhibit neoblast proliferation.

Freshwater planarians are a promising model organism to monitor environmental pollutants and assess impact through in vivo experiments. Indeed they have already been used to assess toxicological effects of diverse substances such as metals, biocides and insecticides, pharmaceutically active compounds, surfactants and other organic and inorganic contaminants (Li 2012; Horvat et al. 2005; Zhang et al. 2015). Knakievicz & Ferreira (2008) investigated the effects of cooper (Cu^{2+}) on the flatworm *Dugesia tigrina* by evaluating mobility, regeneration performance and reproductive performance. The results showed that mobility and time for regeneration was significantly affected by Cu^{2+}. It was observed serious impairments in locomotion and a delay on the appearance of eyespots and auricles in regenerating was observed when compared to non-exposed organisms. Also, chronic exposure effects evidenced a reduction in fecundity and fertility rates of *D. tigrina*. Horvat et al. (2005) detected behavioural and morphological changes observed in flatworm *Polycelis felina* exposed to herbicide norflurazon. Not only it was observed locomotion disorders but also depigmentation tissue, body deformations and shortening of auricles. Ofoegbu et al. (2016) studied the toxicity of tributyltin (TBT) using the freshwater planarian *Schmidtea mediterranea* and observed a dose dependent reduction on food intake and locomotor activity due to failure to sense and capture preys.

1.7. Main objectives and relevance of the study

The objective of the present work is to understand the effects of AgNP of different size in comparison with ionic form $AgNO_3$ using behavioural endpoints of planarian *Dugesia tigrina* after acute and chronic exposure. This rationale is based on the fact that AgNP are currently one of the most widespread used nanomaterials and information

about its safety and potential toxicity to aquatic environment is needed. To carry out the objective of this work, planarians *Dugesia tigrina* were exposed to AgNP of 10-25 nm, AgNP 3-8nm and AgNO3. The parameters chosen to estimate the toxicity were the survival, feeding activity, locomotion activity and head regeneration.

1.8. Thesis organization

This thesis is divided in three chapters:

Chapter 1: a) the general introduction and state of the art regarding silver nanoparticles toxicity and presence in the environment and 2) describing and presenting planarians and the species *Dugesia tigrina* as a model species in ecotoxicology;

Chapter 2: the scientific paper “Toxicity of silver nanoparticles and silver nitrate to the freshwater planarian *Dugesia tigrina*”;

Chapter 3: a general discussion and conclusions are presented, highlighting the main achievements of this thesis.

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Chapter 2

Toxicity of silver nanoparticles and silver nitrate to the freshwater planarian *Dugesia tigrina*

2. Toxicity of silver nanoparticles and silver nitrate to the planarian *Dugesia tigrina*

2.1. Introduction

Over the last years, silver nanoparticles (AgNP) have received remarkable attention in several areas of industry and within the scientific community (Abbasi et al. 2014). First of all, AgNP hold great promise in electronics, energy and medical areas due to their unique properties when compared to their counterparts such as high electrical and thermal conductivity, chemical stability and catalytic activity (Ahamed et al. 2010; Buzea et al. 2007). Also, AgNP are known for their powerful antimicrobial activity making them valuable components in a variety of consumer products used in everyday life such as clothes, food packages, cosmetics, lotions and paints (Rai et al. 2009). Despite the benefits derived from AgNPs application, their production and consumption lead to their increasing release into the environment with potential risks to human health and biota (Fabrega et al. 2011; Benn & Westerhoff 2008). AgNPs can reach aquatic systems by the release of effluents from waste water treatment plants, runoff from agricultural soil, roads, or urbanized areas, or by accidental spillage (Markus et al. 2016). Once in the environment, AgNP are prone to suffer several physicochemical processes that influence their bioavailability, fate and toxicity in aquatic systems (Levard et al. 2012). It is generally accepted that intrinsic properties of AgNP such as shape, size, coating surface or synthesis method along with environmental factors such as pH, temperature, ionic strength or dissolved organic matter have an important role on the behaviour and effects of AgNP to aquatic ecosystems (Lu et al. 2010; Liu & Jiang 2015; Tran et al. 2013). Many studies have been undertaken to better understand the toxicity of AgNP in the environment involving several organisms of different groups, for example, the bacteria *Pseudomonas putida* (Matzke et al. 2014), the aquatic snail *Physa acuta* (Gonçalves et al. 2016), the fish *Danio rerio*, the algae *Pseudokirchneriella subcapitata* (Ribeiro et al. 2014) and terrestrial plant *Lolium multiflorum* (Yin et al. 2011). Most studies suggest that AgNP toxicity is mainly the result of the Ag^+ release, however some authors defend that Ag^+ is not the only explanation for toxicity and AgNP have intrinsic properties that also contribute to toxicity (Fabrega et al. 2009; Auffan et al. 2010).

Nanoparticle's bioavailability and effects are also often related to model organism's life traits. Therefore, using different organisms, with different life traits will improve accuracy of assessments of AgNPs ecological hazards. Freshwater planarians have been gaining interest as a model organism in ecotoxicology to assess effects upon exposure to several compounds (Li 2012; Wu et al. 2014; Lowe et al. 2015). In fact, these organisms exhibit unique biological and behavioural characteristics which highlight their use in research areas such as neurobiology and toxicology (Hagstrom et al. 2015). Besides that, planarians are easy to manipulate and maintain in laboratory conditions. Planarians have a primitive nervous system with a cerebral ganglion connected to nerve cords along the body that coordinates actions and transmits signals to and from different parts of the body. Planarian's auricles are used as chemosensory organs while photoreceptors can detect light direction and intensity, while cilia are used for locomotion. Furthermore, planarians are known for their predatory behaviour once they have the ability to find, attack and capture the prey using mucus which they digest using their pharynx (Vowinckel & Marsden 1971; Takano et al. 2007). Another particular biological set is their regeneration capability which is due to presence of stem cells, called neoblasts. These neoblasts proliferate originating a mass of unpigmented cells, the blastema, that grows and differentiate recovering the missing parts (Kostelecky et al. 1989; Mineta et al. 2003).

Considering the above mentioned, the main goal of the present study was to understand the effects of AgNPs to the freshwater planarian Dugesia tigrina, as a promising organism in nanoecotoxicology. For that two AgNP of different sizes, along with AgNO3 were used for laboratory exposures. Effects on D. tigrina were compared using survival, head regeneration and behavioural endpoints such as locomotion and feeding.

2.2. Material and methods

2.2.1. Test organisms

Dugesia tigrina (sexual strain) were cultured in plastic containers with ASTM hard water (ASTM,1980) medium in the dark, at 20 ± 1 °C and fed once a week with bovine liver (Oviedo et al. 2008). Culture medium was renewed twice a week. Seven days before and during experiments planarians were not fed to avoid interference of food with

experimental procedures. Intact planarians used in the experiments were checked for any morphological abnormalities and measured 10 ± 1 mm total length.

2.2.2. Chemicals

Silver nitrate as a crystalline powder (99% purity; CAS 7761-88-8) was purchased from Sigma-Aldrich. Silver nanoparticles with an initial average particle size of 3-8 nm (further referred as AgNP (3-8 nm)) and 60 nm (further referred as AgNP (10-25 nm)) were supplied by AMEPOX (Poland) dispersed in water with initial concentration of 500 mg L^{-1}. For testing, all test suspensions were prepared in ASTM medium by dilution of initial dispersions of AgNP.

2.2.3. Characterization of silver nanoparticles

AgNP (3-8 nm) were produced by AMEPOX as conductive adhesives for the microelectronic industry. This particle was characterized in ASTM media and described by Ribeiro et al. (2014). The AgNP (10-25 nm) were produced by the same company to be incorporated in conductive ink for ink-jet a pplications and their particle size was characterized in the present study in ASTM media in a suspension of 5 mg/L. This characterization was carried out in a 48h period, with measurements taken at time zero (immediately after dispersion), 24h and 48h after dispersion in ASTM media. Transmission electron microscopy (TEM) after dispersion in water were available by the producers, while dynamic light scattering (DLS) was used to measure the size distribution of the AgNP along with the potential aggregation/agglomeration state in the ASTM media (Malvern Zetasizer).

2.2.4. Acute toxicity tests

Organisms were exposed for 96 h to different concentrations of $AgNO_3$ (50 to 850 µg/L), AgNP (10-25 nm) (25 to 400 µg L^{-1}) and AgNP (3-8 nm) (100 to 2400 µg L^{-1}), considering preliminary results obtained in range finding tests (data not shown). Five replicates were set up per concentration with five planarians per replicate, in a plastic container with 40 mL of experimental suspension at 20 ± 1°C in the dark. Immobilisation

was recorded every 24 h. Planarians were considered immobile if disintegrated or no reaction was observed after poking or exposed to intense light

2.2.5. Chronic toxicity tests

Organisms were exposed for 8 days to different concentrations of $AgNO_3$ (10 to 100 µg/L), AgNP (10-25 nm) (10 to 50.6 µg L^{-1}) and AgNP (3-8 nm) (100 to 506 µg L^{-1}). These concentration ranges were also based on preliminary range finding tests (data not shown). Three replicates were performed per concentration and five planarians per replicate, in plastic container with 40 mL of experimental solution at 20 ± 1°C in the dark. After 8 days, individuals were collected from each replicate and different behavioural endpoints were assessed as described below.

2.2.6. Locomotion activity assay

The methodology used to measure planarian's locomotion activity was adapted from Rodrigues et al. (2016) with slight modifications. After the chronic exposure, planarians were allocated into 24-multiwell plates (one planarian in each well) with 1 mL ASTM hard water. Locomotion activity was recorded using the ZebraBox™ apparatus and the ZebraLab® v3 software (Viewpoint, France). Fifteen planarians per concentration were video tracked over 13 min period (with a previous 1 min of acclimation to light conditions). Locomotion activity was calculated by the distance covered (cm) per min during the 13 min observation period.

2.2.7. Feeding activity assay

Feeding activity was measured according to Ofoegbu et al. (2016) with slight modifications. After chronic exposure, 10 planarians per concentration were moved to crystalizing dishes (one planarian in each crystalizing dish) containing 30 mL of ASTM hard water and 30 6-days old *Chironomus riparius* larvae. After 3 h, the number of remaining larvae was recorded.

2.2.8. Head regeneration assay

For regeneration assays planarians were decapitated immediately below the auricles insertion and above pharynx. Decapitation was performed with a scalpel supported by binocular microscope for better cut precision. This was executed in a Petri dish placed on ice to restrain the mobility of the planarians. Each headless animal was immediately exposed to the same chronic range concentrations as described above. Exposure was carried out in 6-multiwell plates with 5 mL of experimental suspension in each well. Animals were exposed during 8 days and head regeneration was followed. Ten replicates (1 planarian per well) per concentration were used. Each replicate was examined every 24 h under Zeiss stereo microscope (KL 300 LED) to follow the regeneration process. Results were reported as days necessary for regeneration of photoreceptors which in normal conditions take place within four days after decapitation.

2.2.9. Statistical analysis

The effects of AgNPs and $AgNO_3$ exposure to *Dugesia tigrina* were assessed by a one-way analysis of variance (ANOVA) followed by multiple comparison Dunnett's post hoc test, to depict significant differences between treatments and the control treatment ($p<0.05$). These calculations were performed with GraphPad Prism version 6.0 for Windows. The LC50 values were calculated by GraphPad using the best nonlinear regression curve fit, which was a sigmoidal, four parameter logistic regression.

2.3. Results

2.3.1. AgNP characterization

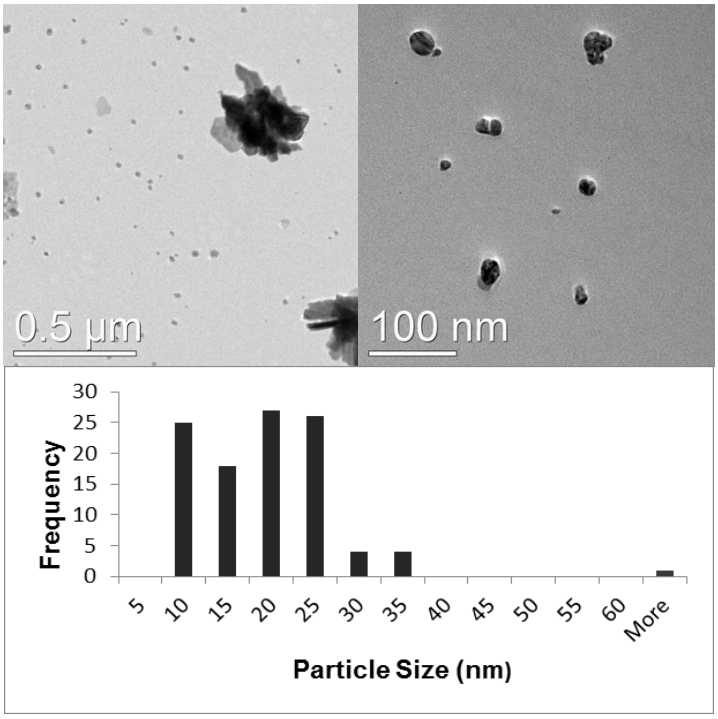

Figure 5. Particle size distribution and TEM images of AgNP (10-25 nm) dispersed in distilled water using TEM images, with the majority of particles ranging size from 10 to 25 nm.

The characterization of AgNP (10-25nm) in ASTM media by TEM analysis showed that the AgNP claimed by the producers to have a mean size of 60 nm showed in water a wider size distribution, ranging from 10 to 35 nm, being the most frequency from 10 to 25 nm. When this characterization was performed in ASTM media the hydrodynamic diameter measured (Z-average (d.nm)) was higher than in distilled water. At time 0h, particles presented 39.02 ± 3.97 nm, increasing to 66.9 ± 2.25 nm after 48h. This indicates that AgNP (10-25 nm) were agglomerating or aggregating in ASTM. The surface zeta potential was −25 mV at time 0h, and did not change significantly with time (-24.3 mV at 48h) revealing some stability in ASTM media.

The characterization of AgNP (3-8 nm) in a 10 mg/L dispersion was already described by Ribeiro et al. (2014) showing that during 3 days the hydrodynamic diameter in ASTM media was increasing from 80 nm to approximately 350 nm.

2.3.2. $AgNO_3$ and AgNPs acute toxicity

The results obtained in the acute toxicity tests with $AgNO_3$ and AgNP (10-25 nm) are shown in Figure 6 and Table 1.

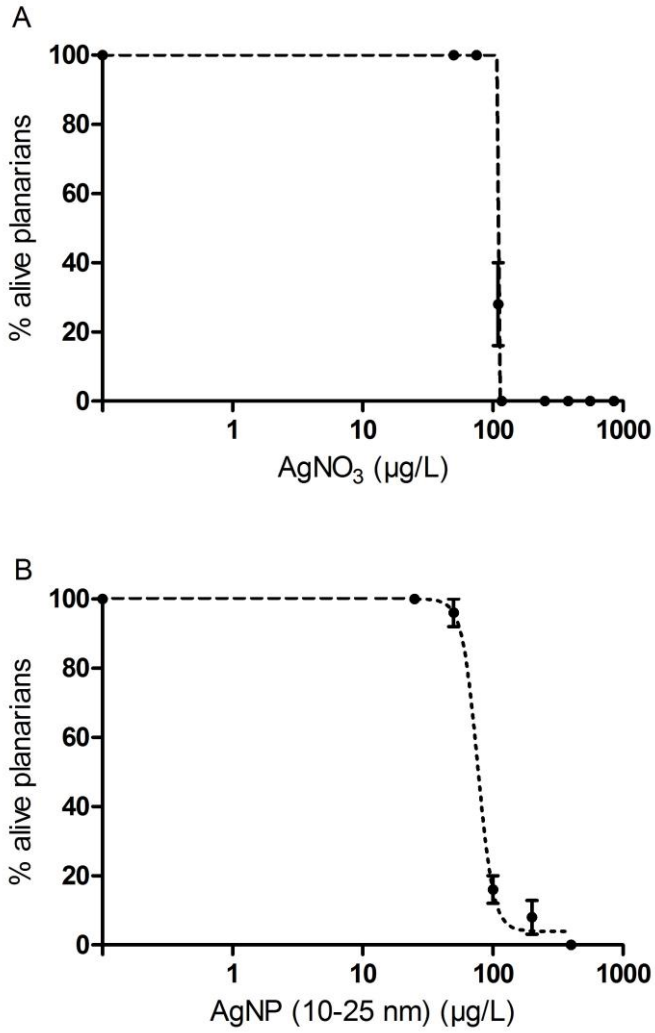

Figure 6. Dose–response curves on survival of *Dugesia tigrina* exposed to (A) $AgNO_3$ and (B) AgNP (10-25 nm) for 96 h in ASTM medium. Data are given as median (50%) response values (µ/L) after sigmoid four parameter logistic regression.

D. tigrina showed same LC50 values for 24-h, 48-h, 72-h and 96 h for $AgNO_3$ of 109.1 µg L^{-1} (80.97-147 µg L^{-1} as 95% CI). LC50 values for $AgNO_3$ were equal over time because there was no variation in mortality during test from the first 24 h of exposure till the 96 h. Further effects besides mortality could be observed during the first 24 h of exposure which included several effects at the head region like head reduction, disintegrated auricles and front part or total head dissolution, particularly at 100 µg L^{-1}. During the test, after 72 h planarians start to regenerate since it was possible to observe formation of the blastema. Regarding AgNPs acute toxicity, similar results were observed for the AgNP (10-25 nm) exposure in comparison with results obtained for $AgNO_3$. A similar pattern was observed for the LC50 in time, presenting a 24 h, 48 h, 72 h and 96 h LC50 of 76.62 µg L^{-1} (89.60-102.7 µg L^{-1} as 95% CI). Head dissolution followed by head regeneration was also observed at concentrations of 100 µg L^{-1} and 200 µgL^{-1}. For AgNP (3-8 nm), mortality or head dissolution was not observed during test even at the highest concentration tested (2400 µg L^{-1}).

Table 1. Summary of the toxicity endpoints derived from all tested Ag forms exposure on mortality, locomotion and feeding activity of *D. tigrina*. Results are expressed as mean with 95% CI. Data is expressed as µg L^{-1}. NOEC = no-observed-effect concentration; LOEC = lowest-observed concentration; n.m.= no mortality observed.

	Acute toxicity test		**Locomotion activity**		**Feeding activity**	
Substance	**48 h-LC50**	**R^2**	**NOEC**	**LOEC**	**NOEC**	**LOEC**
$AgNO_3$	109.1 (80.97-147)	0.97	75	100	40	50
AgNP (10-25 nm)	72.67 (89.60-102.7)	0.97	22.5	33.8	33.8	50.6
AgNP (3-8 nm)	n.m.	-	338	506	338	506

2.3.3. Locomotion test

Figure 7 and Table 1 show the results obtained in the locomotion tests, indicating the distance covered (cm/min) for planarians exposed to a gradient of $AgNO_3$, AgNP (10-25 nm) and AgNP (3-8 nm). All Ag forms significantly decreased the locomotor activity of *D. tigrina* when compared to the control treatment. A dose-response relationship was observed with increasing concentrations reflected by shorter distances covered by *D.*

tigrina. The distance covered was significantly decreased by 47.4% after exposure to 100 µg L^{-1} $AgNO_3$ ($F_{(5, 63)}$ = 8.9; p<0.05), by 23.14% and 48.41 % after exposure to 33.8 and 50.6 µg L^{-1} AgNP (10-25 nm), respectively ($F_{(5, 50)}$ = 8.6; p<0.05) and by 45.4% after exposure to 506 µg L^{-1} AgNP (3-8 nm) ($F_{(5, 110)}$ = 14.73; p<0.05).

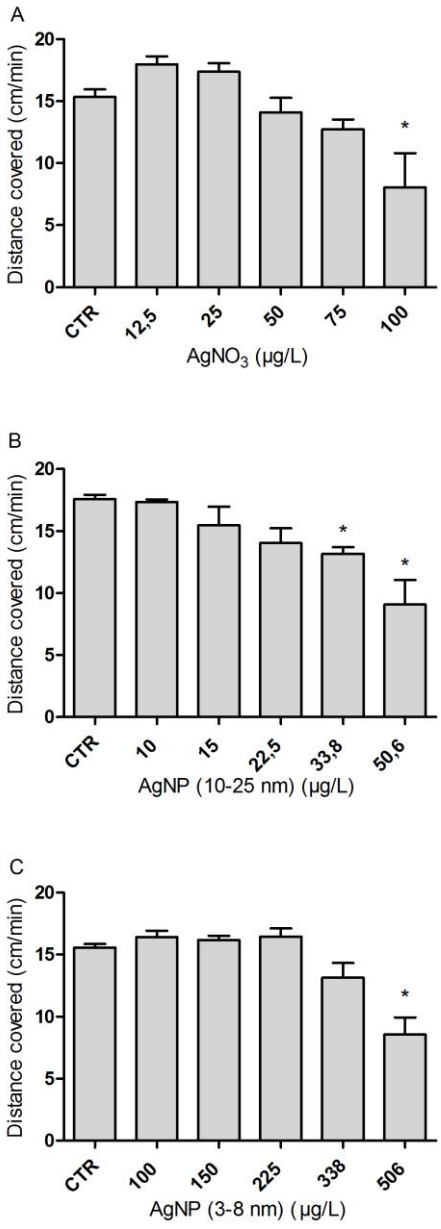

Figure 7. Distance covered (cm/min) by *Dugesia tigrina*, using the automated video tracking system, after 8 day chronic exposure to (A) AgNO3, (B) AgNP (10-25 nm) and (C) AgNP (3-8 nm). Data are expressed as mean values (bars represent standard error). Asterisk denotes a significant difference compared to the control treatment (Dunnett's test, p < 0.05).

2.3.4. Feeding activity

Feeding activity of planarians *D. tigrina* for $AgNO_3$, AgNP (10-25 nm) and AgNP (3-8 nm) is shown in figure 8 and table 1.

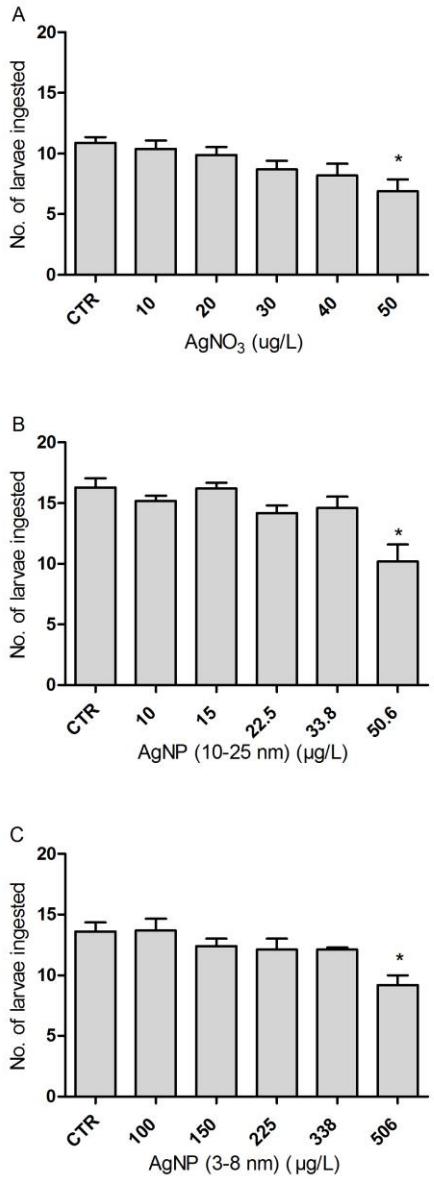

Figure 8. Feeding activity of *D. tigrina*, as number of chironomids larvae ingested in 3 h, after chronic exposure (8 days) to to (A) AgNO3, (B) AgNP (10-25 nm) and (C) AgNP (3-8 nm). Data are expressed as mean values (bars represent standard error). Asterisk denotes a significant difference compared to the control treatment (Dunnett's test, $p < 0.05$).

Similar to locomotor activity, increasing concentrations of $AgNO_3$ and AgNPs tested caused a reduction in feeding activity. The number of larvae ingested was significantly decreased by 37.62% after exposure to 50 µg L^{-1} $AgNO_3$ ($F_{(5, 53)}$ = 3,9; p<0,05), by 37.43

% after exposure to 50.6 µg L^{-1} AgNP (10-25 nm) ($F_{(5, 54)} = 7.3$; $p<0.05$) and by 32.3% after exposure to 506 µg L^{-1} AgNP (3-8 nm) ($F_{(5, 54)} = 4.74$; $p<0.05$).

2.3.5. Head regeneration

Head regeneration measured as days until photoreceptors formation was not significantly altered in planarians exposed for 8 days to all forms of Ag tested ($p>0.05$). It was observed a new formation of photoreceptors 4 days after decapitation.

2.4. Discussion

The mode of action of AgNPs in different biota and which forms are responsible for toxicity are a complex and challenging issue. Many studies have been undertaken to investigate whether the toxicity is related to nanoparticles themselves or to the dissolved ions (Liu & Jiang 2015). Therefore, the aim of the present work was to understand the acute and sub-lethal effects of AgNPs with different size ranges in comparison with $AgNO_3$ to the freshwater planarian *D. tigrina*.

According to the 24-h LC50 values for *D. tigrina*, AgNP (10-25 nm) presented higher acute toxicity than $AgNO_3$. In contrast, AgNP (3-8 nm) did not cause mortality in planarians during experiment representing the least toxic nanoparticle in the present study. Ribeiro et al. (2014) reported that the toxicity of the same AgNP (3-8 nm) was lower than $AgNO_3$ to *Daphnia magna* and *Danio rerio*. It is important to realize that this result contradicts the fact that smallest nanoparticles are in general more toxic than largest ones. AgNP (3-8 nm) could present aggregates decreasing their volume to surface ratio and therefore reducing the rate of Ag ion release in ASTM media and consequently present lower toxicity. Kustov et al. (2014) estimated the toxicity of AgNP using *D. tigrina* and observed planarians death at 24-h at concentration of 10 µg L^{-1} of $AgNO_3$ and a colloidal silver solution (9-15 nm) which is less than the concentration reported in the present study. This difference in toxicity towards the exposure to $AgNO_3$ and AgNP can be explained by the smaller planarians (length) used by Kustov et al. (2014) which are expected to be more sensitive than larger planarians.

Moreover, in the first 24 h of exposure, planarians experienced morphological changes in the head region such as auricles disintegration and head dissolution. Previous

studies suggest that some metals accumulate in the head region increasing its concentration when compared to tail region, consequently causing physiological dysfunction in the nervous system (Wu et al. 2011). Head dissolutions have been described before for planarians exposed to cadmium (Calevro et al. 1998). After 72 h of exposure it was observed a new formation of the blastema in the head region meaning that neoblasts were active and proliferating. Regeneration in planarians involves proliferation of stem cells (neoblasts) to form new tissues (blastema) and remodelling old tissues to complete the normal size. Interestingly, results showed that head regeneration was not a sensitive endpoint in *D. tigrina* for all Ag forms tested. Apparently, neoblast cells responded adequately to wounding signals induced by Ag in *D. tigrina*. Contrarily, Kustov et al. (2014) demonstrated effects of a colloidal silver solution (9-15 nm) in *D. tigrina* regeneration in which regeneration was significantly supressed at 10 µg L^{-1}.

Chronic exposure of all Ag forms tested presented a significant effect in planarian's locomotion and feeding activity. Overall, AgNP (10-25 nm) and $AgNO_3$ demonstrated to be the most toxic Ag form in comparison with AgNP (3-8 nm). Generally, toxic substances cause a physiological response in organisms which ultimately cause a behavioural response. Both feeding and locomotion activity showed to be sensitive endpoints. Thus, these behavioural changes may be used to assess toxic effects of nanoparticles. Many researchers have reported similar behavioural effects in planarians exposed to other metals. Kovačević et al.(2009) tested the effects of aluminium to the planarian P*olycelis felina* and reported locomotive disorders at 200 mg L^{-1} of $Al_2(SO_4)_3 \times 18H_2O$ and total lack of movement at 1100 mg L^{-1}. Wu et al. (2014) exposed the planarian *Dugesia japonica* to cadmium and reported a reduction in their locomotor velocity at 5 µM concentrations suggesting that reduced locomotor activity is due to high concentrations of toxicant in the nervous system of the organisms. As mentioned before, Ag could be interfering with the nervous system which is important to sense, locate and capture preys and therefore inhibiting the locomotion and predation behaviour of *D. tigrina*. Feeding activity in the planarian *D. japonica* was assessed by Zhang et al. (2015) and observed a decrease in feeding rates at 1 mg Pb^{2+} L^{-1}.

Comparing the present values of LC50 and LOEC with other freshwater organisms (Table 1), *D. tigrina* demonstrated a comparable sensitivity as values were on the same order of magnitude (µg L^{-1}). For algae *Pseudokirchneriella subcapitata* was reported a EC50 of 190 µg L^{-1} for a AgNP of 20-30 nm (Griffitt et al. 2008) and 32 µg L^{-1} for AgNP 3-8 nm exposure (Ribeiro et al. 2014); For invertebrates such as snails *Physa acuta* were

reported a LC50 of 81.6 µg L^{-1} (Gonçalves et al. 2016); for *Daphnia magna* was reported a EC50 of 187 µg L^{-1} (Asghari et al. 2012) and 11.02 µg L^{-1} (Ribeiro et al. 2014) for suspended powder AgNPs and AgNP 3-8 nm exposure, respectively; Blinova et al. (2013) repoted a EC50 of 20 µg L^{-1} for AgNP (<100 nm) to aquatic crustacean *Thamnocephalus platyurus*; for zebrafish *Danio rerio*, was reported effects on hatching at 40 µg L^{-1} and 100 µg L^{-1} for AgNP 3-8 nm and $AgNO_3$, respectively (Ribeiro et al. 2014). However, regarding mortality, planarians showed no sensitivity to AgNP (3-8 nm) exposure. Hence it is important to have a comparison between different model organisms to better represent the possible effects on biota and to find appropriate concentration thresholds.

Although $AgNO_3$ toxicity has been clearly attributed to the free ion Ag^+, the source toxicity of AgNP is unclear (Levard et al. 2012). Toxicity of AgNP to *D. tigrina* can be explained by a combination of release of Ag^+ from nanoparticles to ASTM media and especially to the intrinsic properties of AgNP (Lopes et al. 2016). Both sources have the potential, for instance, to generate reactive oxygen species or interact with vital cellular enzymes causing morphological changes or death to *D. tigrina* (Seitz et al. 2015b). When in ASTM AgNP (3-8 nm) tend to form aggregations decreasing Ag^+ dissolution due to lower surface area to volume ratio while AgNP (10-25 nm) demonstrate great stability in ASTM. In comparison to $AgNO_3$, AgNP (3-8 nm) showed to be less toxic and AgNP (10-25 nm) was to some extent more toxic. Choi et al. (2008) also report that ionic silver was more toxic than AgNP. Taking this into consideration, the negative behavioural effects of *D. tigrina* after exposure to AgNP could be mainly attributed to the properties of nanoparticles themselves and not only to Ag^+ ions release.

The ecological hazard of a substance is determined by its persistence, bioaccumulation and toxicity. Ag is known to be persistent in the environment, it is one of the most toxic metals to many organisms and has a strong tendency to bioaccumulate in organisms (Luoma 2008). In some cases, it has been observed a low excretion potential in some organisms (Tourinho et al. 2016). Behavioural effects in *D. tigrina* related to AgNP exposure observed in this study can thus have potential ecological impacts in planarian populations.

2.5. Conclusion

To sum up, the results of the present study demonstrate that $AgNO_3$ and AgNP (10-25) presented higher acute toxicity to the planarian *Dugesia tigrina*, when compared to the results from AgNP (3-8 nm) exposure, where planarians were able to survive at higher levels. Locomotion and feeding activity were significantly affected similarly by all Ag forms tested. The main highlight of the present study is that AgNP (10-25 nm) presented the lowest NOEC and LOEC for feeding activities when compared to the other two forms, contradicting most ecotoxicological findings where it is consensual that Ag^+ usually induces higher toxicity than nanoparticles.

Planarian species such as *D. tigrina* are sensitive model organisms suitable for the assessment of behavioural alterations induced by chemical stress and can contribute to the improvement of hazard assessment to be included in risk assessment procedures. They can be used as model organisms for higher trophic levels, and be used as predators, replacing in some cases fish models. This can provide an important input for the 3R's strategy, and reduce vertebrate testing.

2.6. References

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Chapter 3

General discussion and conclusions

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3.1. General discussion and conclusions

AgNPs are nowadays an important class of nanomaterials widely used in several applications. AgNPs have been applied in everyday products such as clothes, lotions and paints. Also, the AgNPs of the present study are used in electronics and microelectronics industries as conductive materials. Given the background, and the lack of ecotoxicity data concerning ecological effects of nanomaterials in the aquatic environment and effects towards non-model organisms the present work aimed to understand the effects of different AgNPs using the freshwater planarian *Dugesia tigrina*. Moreover, read-across is a process claiming to be used also in nanomaterials, and those decisions have to be supported by scientific and regulatory evidences. Acute tests showed that planarians are sensitive to Ag. The AgNP (10-25 nm) revealed to be the most toxic particle followed by $AgNO_3$ and AgNP (3-8 nm). Also, it some morphological alterations namely head dissolution were observed in planarians exposed to. Regarding chronic tests, locomotion and feeding activity were assessed after 8 days of exposure. It was observed significant reduction in locomotion and feeding activity for all forms of Ag tested. Results also showed no responses in terms of head regeneration of planarians exposed to $AgNO_3$ or AgNPs.

More studies regarding AgNP toxicity need to be conducted for a better understanding of AgNP effects in planarians. Studies with different metals suggest that planarians, when exposed to metallic compounds tend to accumulate them in the head region and consequently causing their death by nervous system impairment (Wu et al. 2011). This point was discussed above and since planarians presented head dissolution and given its reduced locomotion and feeding activity, there is a chance that silver accumulates in greater quantity in the head region than in the tail (posterior region). Therefore, to complement this work, a comparison between Ag concentrations in head region and the rest of planarian body could be carried out to understand if Ag toxicity is related to failure of nervous system. For instance, the metallothionein assay would allow to detect and measure presence of Ag in planarians. Metallothionein is a molecule responsible for regulating essential metal ions and detoxifying xenobiotics and non-essential metals. Its expression is induced by bivalent metals and usually it is used as a

biomarker of heavy metals in aquatic organisms. Acetylcholinesterase (AChE) plays an important role in functioning of nervous system and it is very often target of neurotoxic metals. Performing a AChE assay would permit to check signs of neurotoxicity caused by Ag once inhibition of AChE activity can indicate neurotoxicity. Also evaluating a possible alteration of oxidative status by Ag could provide interesting insights on Ag mode of action. Evaluating glutathione levels would indicate oxidative stress caused by Ag.

The head regeneration assay test did not show results in *D. tigrina* as delay in photoreceptors appearance was not observed. This demonstrates robustness and power of neoblasts cells upon Ag exposure. These results were not observed in studies with copper where it was demonstrated delays in regeneration of *D. tigrina* after Cu^{2+} exposure (Knakievicz & Ferreira 2008). By all means, it cannot be concluded that the delay in appearance of the photoreceptors in planarian is an accurate parameter. In fact, Kustov et al. (2014) exposed planarians of the same species to a colloidal solution of 9-15 nm AgNP, where regeneration performance was assessed by measuring area of blastema using computer-assisted *in vivo* morphometry. It was calculated a regeneration index (blastemal area / total body area) as a quantitative measure of cell proliferation. It was found out that regeneration was significantly suppressed. Therefore, this method could be integrated in the present work for more precise results regarding regeneration process of planarian.

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