Universidade de Aveiro Departamento de Biologia

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Ecotoxicological assessment of engineered nanoparticles in *Chironomus riparius*.

Análise ecotoxicológica de nanopartículas sintéticas em *Chironomus riparius*.

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Ecotoxicological assessment of engineered nanoparticles in *Chironomus riparius*.

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 da Doutora Susana Patrícia Mendes Loureiro (Investigadora Auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro).

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palavras-chave

Nanoparticulas de prata (AgNPs), *Chironomus riparius*, toxicidade aguda, toxicidade crónica, testes multigeracionais, biomarcadores, ecotoxicogia sedimentar

resumo

As nanoparticulas e nanopartículas de prata (AgNPs) sempre estiveram presentes no ambiente natural, contudo novos desenvolvimentos tecnológicos tornaram a sua presença ubíqua. Estas irão estar presentes principalmente em águas e depositar-se em sedimentos, cada vez em maiores quantidades à medida que a sua libertação para os ecossistemas prossegue. Para determinar a toxicidade tanto em águas como em sedimentos contaminados, foi escolhida a espécie *Chironomus riparius* tendo sido comparada a toxicidade das AgNPs com o seu semelhante iónico ($AgNO_3$). Em água contaminada os dados analisados foram: eclosão, mortalidade em diferentes estadios, taxas de crescimento, peso e dados da emergência. A eclosão de massas de ovos teve valores de EC_{50} de 3882.27 $\mu g.L^{-1}$ e 77.68$\mu g.L^{-1}$, para AgNPs e $AgNO_3$, respetivamente. A mortalidade no primeiro estadio larvar registou valores de LC_{50} para AgNPs de 1026.94 $\mu g.L^{-1}$ e de 23.37 $\mu g.L^{-1}$ para o $AgNO_3$. As taxas de crescimento tanto para a cabeça como para o corpo não sofreram alterações. O tempo médio para a emergência sofreu alteração com um valor calculado de EC_{50} de 1289.86 $\mu g.L^{-1}$ e a emergência o EC_{50} de 843.71 $\mu g.L^{-1}$ em relação apenas a AgNPs. Os resultados demonstram que os ovos são uma fase mais resistente do ciclo de vida. Um ponto crítico de sensibilidade para com contaminantes na coluna de água é atingido quando ocorre a metamorfose. Os possíveis efeitos de compostos de prata no sedimento foram avaliados através da análise da sobrevivência, taxas de crescimento, peso, fertilidade, rácio sexual e os biomarcadores: catalase, acetilcolinesterase, glutatione-S-transferase e peroxidação lipídica. As taxas de crescimento para a cabeça e corpo foram afetadas tendo um EC_{50} de 222.62 $mg.Kg^{-1}$, e 229.84 $mg.Kg^{-1}$, respectivamente para a exposição com AgNPs. A sobrevivência das larvas quando expostas a AgNPs foi afetada, com um LC_{50} de 334.03 $mg.Kg^{-1}$ e o tempo para emergir com um EC_{50} calculado de 122.19 $mg.Kg^{-1}$. Apenas foram registadas alterações na atividade das enzimas em relação à catalase e à glutationa-s-transferase, com um aumento da atividade das mesmas. O número de massas de ovos depositados diminui com o aumento da concentração de AgNPs, com um EC_{50} de 13.10 $mg.Kg^{-1}$ enquanto na fertilidade dessas mesmas massas de ovos registou-se um EC_{50} de 70.21 $mg.Kg^{-1}$.

A emergência de total de adultos da geração filial registou um EC_{50} de 49.20 $mg.Kg^{-1}$. Os dias médios para a mesma, também foi sujeito a impactos de toxicidade e foi calculado um EC_{50} de 24.50 $mg.Kg^{-1}$. A contaminação do sedimento com AgNPs demonstrou causar efeitos adversos tendo possíveis repercussões intergeracionais.

keywords Silver nanoparticles (AgNPs), *Chironomus riparius*, acute toxicity, chronic toxicity, life cycle, sediment, and water toxicology, biomarkers

abstract Nanoparticles and silver nanoparticles (AgNPs) have been present in natural environments nevertheless new developments have made their presence ubiquitous. They will majorlly be present in waters and sediments with increasing rates as the release to the ecossystems proceeds. Toxicity was assessed and compared in both contaminated water and sediment, the midge species *Chironomus riparius* was chosen and the toxicity of AgNPs and its ionic counterpart ($AgNO_3$) were analysed and compared. In spiked water the endpoints assessed were: hatching , survival in different stages, larval growth, weight and emergence. Hatching was significantly affected when exposed to AgNPs and $AgNO_3$ spiked water with EC_{50}s values of 3882.27 $\mu g.L^{-1}$ and 77.68 ± 6.07 $\mu g.L^{-1}$, respectively. First instar mortality in acute exposure had a value of LC_{50} for AgNPs of 1026.94 $\mu g.L^{-1}$ and for $AgNO_3$ of 23.37 $\mu g.L^{-1}$. Both head and body length growth rate were unresponsive. Mean time of emergence had an EC_{50} value of 1289.86 $\mu g.L^{-1}$ and EC_{50} value for emergence the of 843.71 $\mu g.L^{-1}$ in regards only to AgNPs.The results for Ag showed that eggs may be a high resistant state in the chironomids life cycle. A critical point of low resistance to water borne contamination is atained during metamorphosis. To evaluate the toxicity of silver compounds when present in sediment endpoints for survival, larval growth, emergence, weight, fertility, sex ratio and biomarkers: catalase, acetylcholinesterase, glutathione-S-transferase and lipid peroxidation were chosen. Growth rates of head and body had an EC_{50} of 222.62 $mg.Kg^{-1}$, and 229.84 $mg.Kg^{-1}$, respectively for AgNPs exposure. Regarding AgNPs exposures, survival of 10 day larvae showed a LC_{50} of 334.03 $mg.Kg^{-1}$ and time to emerge a EC_{50} of 122.19 $mg.Kg^{-1}$. An increase in activity was only observed in catalase and glutathione s-transferase. The number of egg masses had an EC_{50} 13.10 $mg.Kg^{-1}$ and their fertility an EC_{50} of 70.21 $mg.Kg^{-1}$. Total emergence of adults had EC_{50} of 49.20 $mg.Kg^{-1}$ and the time to emergence was significantly affected in the filial generation. Sediment contamination with AgNPs proved to be detrimental to *C.riparius* with possible intergenerational consequences.

Index

List of figures and tables

List of figures

Chapter 1

Chapter 2

Chapter 3

List of tables

Chapter 2

Chapter 3

Chapter 1

General Introduction

1. General Introduction

1.1 Nanoparticles – History and Ubiquity

Nanomaterials are defined as any material with at least one dimension between 1 and 100 nm and with a specific surface area by volume greater than 60 m^2/cm^3 (European Commission, 2011). However, in ecotoxicology, materials are also considered to be in this category when particles or aggregates are between 100 nm and a few hundred nanometres (Handy and Shaw 2007). Materials can be grouped through their number of nanoscale dimensions; when nanoscale is present in only one dimension, we are in the presence of films, when with two dimensions materials are considered as tubes or fibres, and finally when all three dimensions are nanometric they are considered as true particles (Vert et al. 2012).

Nanoparticles (NPs) have always existed in the natural environment and have been used, most of the times unknowingly, throughout human history in different manufactured products. In the natural environment NPs are ubiquitous and present in the geosphere, hydrosphere and atmosphere. Some authors even consider viruses and bacteria as nanometric entities, as they encompass sizes that range between 10-400 nm and 30nm to 700µm to viruses and bacteria, respectively (Yamada, 2004 and Masciangioli and Zhang, 2003).

Within the geosphere it was realised that soils have a complex matrix of mineral particles and colloids (Reid et al., 2000) and soils with volcanic ash contain silica NPs (Lee and Richards, 2004). Throughout the hydrosphere, NPs were found in several water systems, including: oceans, surface waters, groundwater, atmospheric water and even treated drinking water. These NPs encapsulate a wide variety of nanoscale mineral particles and humic substances (Lead and Wilkinson, 2006 and Wigginton et al., 2007). Moreover, the ocean surface microlayer contains carbon, calcium carbonate, colloids and nano-sized phytoplankton, important in the transport of material in the air-water interphase (El Nemr and Abd-Allah, 2003 and Obernosterer et al., 2005). Additionally at the polar caps, ice cores with over 10,000 years were found to contain carbon nanotubes and carbon fullerenes.

These NPs were probably originated from wildfires across the globe, which occurred naturally, being lighting strikes their major cause for ignition, and spreading particulate matter by thousands of square kilometres (Murr et al., 2004). Small particles, such as these, when present in the atmosphere are known as aerosols and approximately 90% are of natural origin (Taylor, 2002), playing a crucial role in scattering solar radiation away from Earth (Houghton, 2005). It is estimated that 50% of aerosols present in the troposphere are the result of dust storms, (which uplifts materials abraded and refined by erosion, from deserts) while in stratosphere, the mass of these particles amounts to 99% of the total present (d'Almeida, 1989); these materials can incorporate pollutants and most of the times transport microorganism therein (Taylor, 2002). Additionally the smaller a particle is, the farther it will be transported, and the longest its life span in the atmosphere will be. Amongst the other natural components of aerosols present in the stratosphere, in 1985 it was reported the presence of Bismuth oxide NPs, which was related to volcanic eruptions in the beginning of the 1980s (Rietmeijer and Mackinnon, 1997). The volumes of volcanic plumes contain up to $30x10^6$ tons of ash, with individual particles capable of reaching high altitudes (Langmann et al., 2012). In extraterrestrial environment NPs are also conspicuous. The dust proportion in the lunar regolith has nanoparticles, with magnetic properties, in more than 50% of its composition (Taylor et al., 2005). Extraterrestrial bodies were also the trigger for the formation of historical sediments on planet Earth, for instance the abundance of silicate NPs in the Cretaceous-Tertiary boundary, whose presence is probably the outcome of a meteorite impact (Verma et al., 2002).

In human history, NPs have always been used in a wide range of applications. One of the earliest uses was in glazes for Chinese porcelain. In the Roman Empire a goblet called Lycurgus Cup, made in the 4th century A.D., used nano-gold clusters to create different colours depending on the direction of light (Holister et al., 2003). In medieval times NPs were also used in the stained Glasses of Sainte-Chapelle, Paris installed in the 13th century. These consist of a colourless glass substrate covered by a 50–500 μm thin glass layer with a dispersion of metallic NPs, particularly copper and silver (Colomban and Tournié, 2007). Beginning in 1000 A.D. and throughout seven centuries, swords made of Damascus steel were renowned and gathered worldwide appraisal for their hardness and durability.

Nowadays, by analysis on the ancient swords it was found that they contain a composite of nano-sized iron carbide layers intertwined with iron creating a matrix, the complexity and the many layers give the blades their reputed duress (Kobasko, 2011).

Despite the several historical uses, it was only in modern times that it became technologically feasible to mass produce NPs, from the increasingly achieved advances in the fields of physical chemistry, quantum physics, material sciences, biochemistry, and metrology. The availability of NPs has created a deep interest in their study and potential applications by the industry and the scientific community. Private funding for research in nanotechnology is already the most significant, which leads to an increasing incorporation into use of NPs and nanomaterials (Christian et al., 2008 and Pidgeon et al., 2011).

1.2 Nanoparticles – Synthesis

Nanotechnology has the potential to revolutionize materials applied in industry, medicine, agriculture, information technology, etc.; and there are already more than 35 countries with running technological agendas focused on nanotechnology. Currently NPs are designed with specific physico-chemical properties linked with their future application which are ever far reaching and increasing (Aitken and Tran, 2004).

There are two main approaches to synthesize NPs. The “top-down” method consists of manipulation of physical tools to shape materials into the desired shape, size and order. These tools include: laser-beam processing, mechanical and lithographic techniques, the latter the most common. Within mechanical techniques there are methods such as: grinding, allowing and polishing; whilst amongst lithographic techniques, there are processes which use UVs, electron or ion beams, scanning probes and optical near field. Systems that manipulate chemical properties of molecules to induce self-assembly can be considered “bottom-up”. This encompasses methods such as chemical synthesis, chemical vapor deposition; gas phase processes (flame pyrolysis, high temperature evaporation and plasma synthesis), self-assembly, colloidal aggregation, laser-induced assembly, film

deposition and growth, etc. Each technique has its handicaps and benefits, nevertheless it is predicted that “bottom-up” approaches will be more prevalent in the future, as they are more energy efficient, and can synthesize smaller particles with extreme precision, and high reproducibility (Mafuné et al., 2000).

Engineered NPs can be subdivided based on their characteristics, existing two main categories accounting on the number of materials used: single or multiple materials. Other subdivisions exist within the multiple material category such as core/shell and composite/multilayer NPs (Kim et al., 2004 and Wu et al., 2007). As an example of single material NPs, there is the case of carbon-based materials such as Buckminsterfullerenes, which consist of carbon spheres with 60 atoms; and carbon nanotubes (Fagan et al., 1991 and Smith et al., 2007). Composite/multilayer NPs may comprehend particles with different inorganic materials bound together as alloys or several metallic compounds fused together by means of polymers or silica (Van Berkel et al., 2009). Core/shell NPs can be characterized by a central point with a different material than the outside one, which acts as a shell or a coating. These different types of materials can be available in order to fulfil several needs for interactions between organic and inorganic materials. Organic compounds include materials such as citrate, cysteine, carbonate, etc. Additionally surfactants compounds such as sodium dodecyl sulfate can be used to maintain the stability of the colloidal suspension. In due course these arrangements are linked to their final application. NPs can additionally comprehend several shapes such as: spherical, hexagonal, multiple cores, multiple coating, matryushkas-type materials, movable core and hollow shell (Chaudhuri and Paria, 2012).

Materials can be manipulated in a way to enhance their characteristics, leading to products which are designed with specific purposes. For instance the efficiency in catalytic reactions can be boosted through fine-tuning the chemical potentials of NPs by augmentation of the surface to volume ratio. Additionaly, through the use of magnetic nanocomposites the electric capability, resistance, hardness and toughness of metals and alloys can be improved. (Luther, 2004). Along with their potential applications, NPs have already been and are being used in products available in the market such as: new types of construction materials, electronics, cosmetics, optics, textiles, medical devices, food

packaging, among others. Medical devices using nanotechnology are known to potentially improve the ability to deliver specific quantities of nutrients and drugs to target cells. Medical devices can additionally detect toxins and pathogens in low concentrationsand also identify and monitor diseases, through the body fluids (Stylios et al., 2005). In the case of food packaging, nanotechnology has improved to the point of indicating the initial stages of decomposition, and controlling in a more efficient way gas transfers between the packaging and its surroundings. Water treatment technology, fuel cells, biosensors capable of measuring environmental toxic compounds, biocides and agents for environmental remediation, methods of disinfection and self-cleaning and self-disinfecting surfaces, materials and equipment are also new or under development technologies which can significantly improve several processes (Freitas, 2005; Karnik et al., 2005; Alli, 2012; Tugulea et al., 2014; Tesh and Scott, 2014).

Besides the intended synthesis of nanoparticles, there is also an unwillingly production of nanoparticles; which are the by-products of, or induced by human activities. Such particles may also present toxicological risks, explicitly: aerosols created from vehicle, urban, industrial emissions, and fungicidal nanoparticles in the soils. (Handy and Shaw, 2007). In urban areas, the aerosols derived from human action consist mostly of post-combustion products, with as much as 50% of the total mass, comprised of carbon particles with traces of transition metals (Harrison and Jones, 1995; Donaldson and Stone, 2003).

This raises questions about the toxicity of NPs and their effects on life forms, with critics alerting to the potential hazards and optimists focusing on the prospects of application of new types of materials to induce environmental remediation, capable of returning the ecosystems to a more pristine state (Harrison, 2007 and Navarro et al., 2008).

1.3 Nanoparticles – Ecotoxicity

Albeit organisms have been continuously exposed to natural NPs throughout their evolutionary history, there are still potential harmful effects relaed to such exposure

(Fubini and Fenoglio, 2007). Additionally natural NPs, besides having intrinsic potential toxicity, when present in polluted environments, will possibly incorporate toxic materials (Lee and Richards, 2004). When present within biological systems, NPs are capable of inflicting inflammatory, oxidant and anti-oxidant effects, due to their greater surface area per mass compared with larger-sized particles (Oberdörster et al., 2005).

In the atmospheric component of Earth, there are several reported cases of NPs with adverse consequences in biological systems, such as volcanic ashes, which when in contact with the body, are capable of inducing ocular and dermal irritation and respiratory problems if inhaled. (Horwell and Baxter, 2006). Atmospheric pollution with particles less than 100nm has been correlated with several syndromes like coronary and respiratory diseases and tumours, reducing the life expectancy, and ultimately causing death. In 2002 the World Health Organization had already estimated that 800,000 deaths per year, were related to this type of particles, being already the 13th most common cause of death (WHO, 2002). When particles are inhaled they are deposited throughout the respiratory tract by diffusion, and the deposition efficiency is inversely correlated with particle size (Swift and Strong, 1996). Thereafter NPs will suffer transcytosis through cells entering in contact with blood and lymph, easily reaching more sensitive tissues and organs such as spleen, bone marrow, lymph nodes and heart. When present within the circulatory system it is proven that NPs induce accretion of platelets leading to the formation of blood clots particularly in the pulmonary capillaries which improve the risk of thrombosis (Oberdörster et al., 2005).

Nano-size particles can enter the host systems via skin spores, debilitated tissues, injection, olfactory, respiratory and intestinal tracts (Yah et al., 2012). NPs have been demonstrated to be present and induce toxicity in aquatic and terrestrial invertebrates, (Viarengo and Nott, 1993). For instance anurans, with life stage comprising embryos, tadpoles and adults, have an extremely permeable skin which makes them excellent indicators of environmental health, have been shown to be extremely sensitive to NPs toxicity with possible teratogenic effects (Ibarra et al., 2015). NPs have been additionally shown to induce toxicity in fish (Oberdörster, 2004; Asharani et al., 2008), molluscs, (Moore et al., 1997), daphnids (Zhao and Wang, 2012), algae (Suman et al., 2015), soil invertebrates (Tourinho et al., 2012), plants such as wheat (*Triticum aestivum*), oilseed rape (*Brassica*

napus) and *Arabidopsis thaliana* (Larue et al., 2011). Nanoparticles have been show act throught 'direct' mechanisms that require a close contact between nanoparticles and cell membranes, and by 'indirect' influence elicited by the acidity of nanoparticles stabilizing agents. It has been shown that *Escherichia coli* is sensitive to the 'direct' effects of cerium oxide nanoparticles, whereas *Synechocystis* being protected by extracellular polymeric substances preventing direct cellular contacts is sensitive only to the 'indirect' mechanism (Zeyons et al., 2009). In most cases NPS are incorporated through ingestion and endocytosis, this could lead to an overload of endocytosis, triggering cell death and release of pathogens within the cell, increasing the rates of mortality in organisms (Cuervo, 2004).

1.4 Silver nanoparticles and silver ions

One of the NPs with the most usage in various applications and with the fastest growth rate of production are AgNPs, with antifungal and antibacterial properties exploited in several products (Schulz et al., 2002 and Choi et al., 2008). The Woodrow Wilson International Center for Scholars trough the Project on Emerging Nanotechnologies had assembled in 2006 a list of 600 items that contain some form of engineered NPs, which were available for purchase in stores or in the internet (Felcher, 2008). In mid-2014 this number increased to more than 1800, AgNPs were active constituents of air cleaners, air sanitizer sprays, beverages, coatings of refrigerators, condoms, containers, detergents, pesticides, pillows, vacuum cleaners, hair dryers, slippers, socks, face masks, soaps, shampoos, toothpastes, wet wipes. There were also several NP based compounds incorporated into clothing that can act as stain removals, and with sun-blocking and water repellant properties (PEN, 2014).

Metallic compounds are extremely important for the regular functioning of biological systems, nevertheless, as any other chemical, with increasing concentrations they are known to induce toxic effects (Shah, 2003). Silver ions can easily bioconcentrate in living tissues, as they can easily pass through the cell membrane using ion transporters due to their similarities to sodium and calcium ions (Bury and Wood, 1999 and Tupling and

Green, 2002). In humans, exposure is known to trigger argyria, which is a result of accumulation with symptoms of recognizable blue patches in the body tissues (Getler and Rhoads, 1927). As evidence, in other mammals, silver compounds were shown to cause kidney hemorrhage and even low silver concentrations were proven to be hinderers to aquatic life (Scow et al., 1981). Silver compounds have been also proven to be highly toxic to microorganisms ranging from eukaryotes (Kvitek et al., 2009) to bacteria, including 16 of the major strains. In addition it has also been shown that toxicitycan be exacerbated by the use of AgNPs due to the increase in surface area and thus exposure area (Liau et al., 1997 and Prabhu and Poulose, 2012).

Silver exposure can additionally cause a reduction of the energy available to the cell through depletion in adenosine triphosphate, and a deterioration in the cell's exterior layer (Lok et al., 2006). As such silver compounds, especially AgNPs, due to their small size and large reaction surface, have the potential to be developed as a mean to control bacterial infections. However their use could derive in plausible adverse effects on gut microflora, especially when taken orally (Sawosz et al., 2007). Additionally concentrations that affect microorganism are proven to have an adverse effect in components of human cells (Dunn and Edwards-Jones, 2004).

Naturally the silver present in the environment resulted from the weathering of silver rich ore. Silver started to be extracted as a precious metal used with ornamental purposes and eventually its application diversified. During the 20th century, an increase was noticeable in the component of silver in surface waters, which was linked with the result of waste water released by the photographic industry (Scow et al., 1981). Nowadays the extraction of silver amounts to 23 million tons/year and the synthesis the NPs accounts for over 500 tons/year and with a high potential for growth in the future (Köhler et al., 2008). There is a wide scientific unanimity that the utilization of synthetic NPs will pave the way to discharges into ecosystems (Gottschalk et al., 2013). AgNPs can be released to the environment intentionally or unintentionally. Intentional releases can occur whilst using nanoparticle bearing products such as pesticides or as a form of environmental remediation (Lachance et al., 2014). Unintentionally discharges may prove to be the most common; beginning during their synthesis in industrial plants, nanoparticles can also spill

into the environment during incorporation into the goods and while the products are being used and in their eventual demise (Köhler et al. 2008).

The importance of biocidal products containing silver has been recognized since 1998 by the European Parliament. Legislation was approved accounting for the environmental risk assessment of products in the market (European Parliament and Council, 1998). This legislation had a span of 10 years and was further extended in a 2009 Directive (European Parliament and Council, 2009). Likewise, in 2010 the Environmental Protection Agency of the United States issued a declaration to alter the approach to nanomaterials making risk assessment compulsory to every new product, regardless of source material, under the Federal Fungicide, Insecticide, and Rodenticide Act (Code of Federal Regulations, 2010). These laws came to reinforce the need of developing and enhancing the research being made primarily through universities, institutes and governmental agencies, and promote the interchange of knowledge through international and institutional collaboration, oversight and peer review (Chow et al 2005 and Biswas and Wu, 2005). It is worth to point out that until quite recently there was no recurrent analysis of the level of nanoparticles in aquifers and drinking water (Handy and Shaw, 2007), and the literature is still sparse in assessments of concentrations that might be being leached to the environment and present in natural media, thus overlooking one key source of knowledge (Gottschalk et al., 2013; Hassellöv and Kaegi, 2009). This is one of the gaps on the environmental risk and hazard approaches mainly due to the disability of methodologies to detect the presence of particles at ecological relevant concentrations (low mass/low number).

The fate pathway of silver NPs in the environment can be predicted and summarized as shown in Fig. 1.1., becoming apparent the role of aquatic systems and sediments as the ultimate laying basin. The use of products incorporating silver, releases ions and AgNPs into wastewater, and this can be either treated in a sewage treatment plant or discharged into natural waters. Silver which is not removed during this process reaches natural waters via effluents. The sewage sludge/biosolids removed from the sewage treatment plant can be disposed in solid waste landfills, applied to agricultural soils, or incinerated in thermal waste treatment plants. When incinerated, NPs can become airborne; and as the geological

processes set in, will suffer transportation and sedimentation on soils and sediments (Blaser et al., 2008). These materials have the potential to endure and reenter continuously in the geological and hydrological cycle, initially through becoming aerosols by the action of wind erosion, and then incorporating into water droplets or by suffering deposition elsewhere (Mueller and Nowack, 2008).

Thus it is essential to study the effects of AgNPs in the several compartments of the environment and compare it with the findings for the counterpart silver ions as their potential impact in the ecosystem are yet to be fully clarified (Masciangioli and Zhang, 2003).

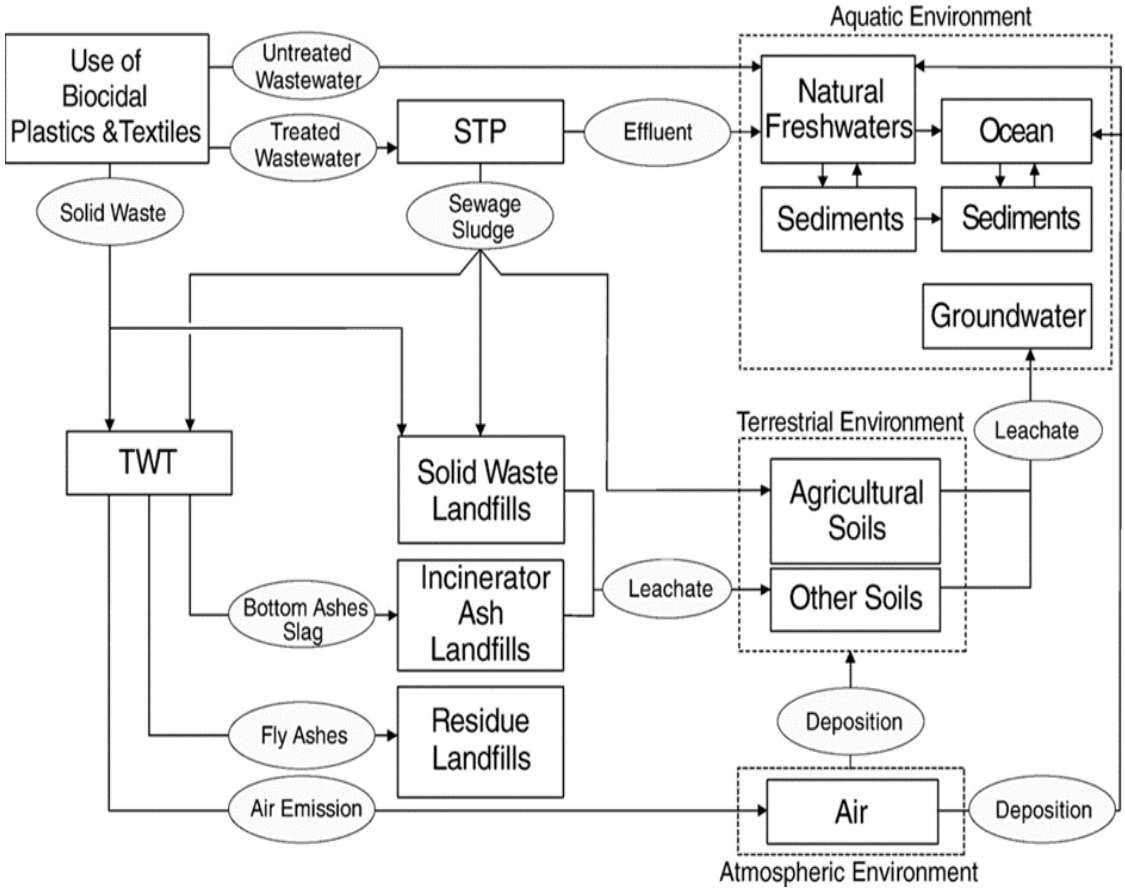

Fig. 1.1. Possible pathway of silver compounds used in biocidal plastics and textiles, when released to the environment as means of solid waste, treated or untreated wastewater. Arrows represent silver flows; STP – sewage treatment plant; TWT – thermal waste treatment (Blaser et al., 2008).

1.5 *Chironomus riparius* and chironomids

The non-biting midge *Chironomus riparius* (Meigen, 1804) is a sexually reproducing detritivore macroinvertebrate organism, common in Portuguese streams. It is widely used in ecotoxicology to monitor the accumulation of metals and insecticides in freshwaters (Vogt et al., 2010 and Rasmussen et al., 2012). The main reasons for their use is that as freshwater insects, chironomids belong to one of the most abundant, species-rich and ecologically relevant taxonomic groups, being represented in climates of the northern hemisphere (Armitage et al., 1995 and Vogt et al., 2007).

This species belongs to a group of particular genera of chironomids *(Procladius ssp., Chironomus ssp., Glyptotendipes ssp.* and *Cricotopus ssp*.) that often become increasingly dominant in contaminated sites (Canfield et al., 1996; Groenendijk et al., 1998; Peeters et al., 2000; De Lange et al., 2005; Marinkovic et al., 2012). The Chironomidae are extremely important organisms in freshwater ecosystems, and they dominate the benthic communities of lotic and lentic environments both in number and in biomass (Merrit and Cummins, 1996; Allan and Castillo, 2007). Chironomids can be found in different aquatic environments due to their capacity to adapt to environments with extreme conditions of pH, temperature, depth and salinity (Armitage et al., 1995). As larvae tend to be extremely selective in their choice site, the number and type of species of Chironomids present, differs wildly between microhabitats (Maasri et al., 2008). Chironomids are present at the basis of the food chain, being detrimental as a food supply to many other major groups of animals, like fish, and therefore the presence of larvae in sediments and species composition is often used as indicators of biological classification of inland water reservoirs (Kirgiz, 1988).

Regarding the species characteristics, *C. riparius* possesses a life cycle which takes approximately 25 days to complete at 20°C. The metabolic rate of larvae is variable and has proven to intensify at higher temperatures and diminishes at lower ones (Vogt et al., 2007). Furthermore their life cycle is subdivided between different media/environmental compartments and split among stages; it is composed of an aquatic phase which includes

eggs, four larval stages (1st, 2nd, 3rd and 4th instar) and pupa whilst metamorphosis occurs (Fig. 1.2.); after pupae exude, chironomids enter the aerial phase commonly referred as adult. The larval stages are the most enduring, accounting for the major part of the life cycle, where the 2nd to the 4th are almost exclusively sediment dwelling. The species in its natural habitat forms large swarms of adults to facilitate reproduction. A few days after leaving the pupal stage, females lay an egg mass which may contain up to several hundreds of eggs. The laid eggs, if fertilized and excluding other aberrant behaviour, will hatch within a few days and a new batch of larvae will hatch and start a new life cycle. (Armitage et al., 1995).

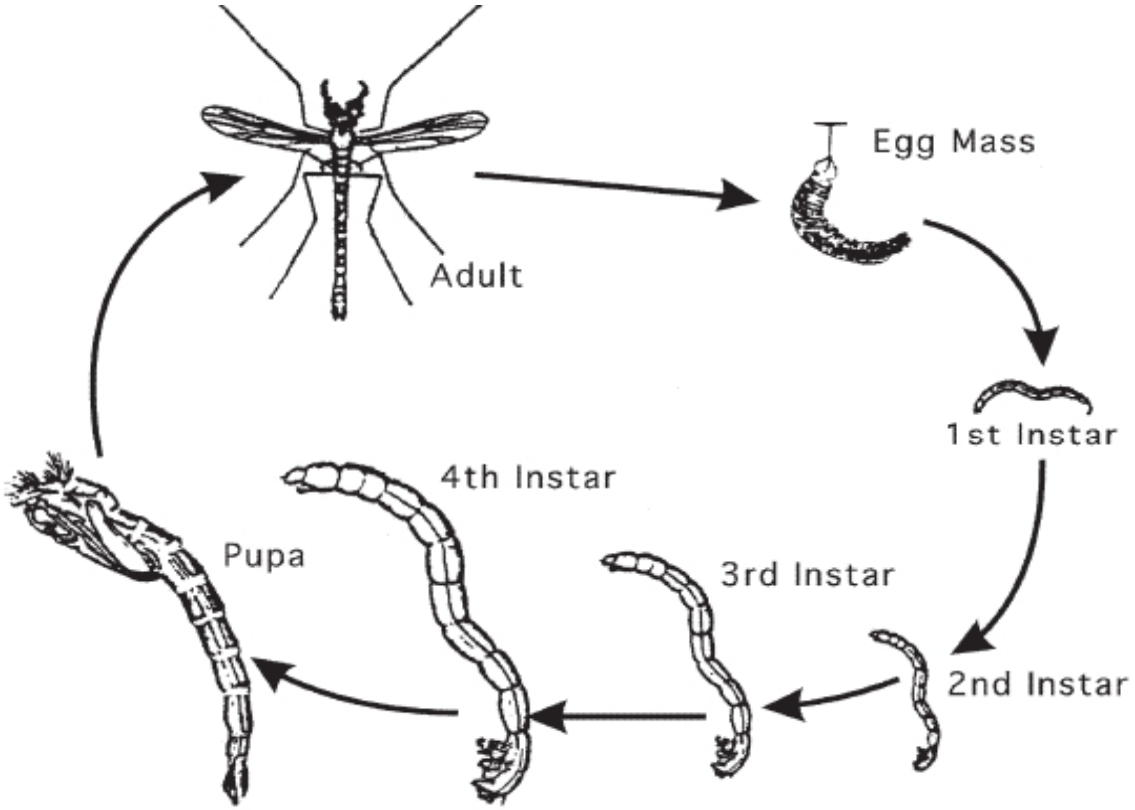

Fig. 1.2. Chironomid life cycle ((adapted from Walker, 1987) in Pixshark).

1.6 *Chironomus riparius* as a test species

It is a widely accepted fact that chemicals are able to move into the aquatic environment by several routes (run-off, drainage and spray drift); therefore it cannot be

overlooked that sediment dwelling organisms can and will be affected by the composition of the overlying water. Besides waterborne contamination, and due to chemicals' sedimentation, the interstitial pore water in the sediment will eventually become in equilibrium with the water column, and chemicals' will additionally be adsorbed to the sediment particles. Thus the use of *C. riparius* allows the assessment of exposure through spiked sediment and spiked water scenarios, unlike most species used in toxicity tests. In their larval stage, individuals of *C. riparius* are in direct contact with sediment. As such they have been used as a model organism to assess sediment toxicity, in their natural environment as well in laboratorial studies, where endpoints as larval behaviour, growth, survival and emergency can be evaluated (Chappie and Burton, 1997; OECD, 2001; Faria et al., 2006; Domingues et al., 2007).

Chironomids have unique characteristics that make them suitable for laboratorial testing. For instance as their life cycle covers three distinct environments: water column, sediment and air, potential contamination assessment can be performedfor each one. Furthermore they belong to the class of insects which is the most represented class in freshwaters, and mainly feed on algae, plants, bacteria or invertebrates during their larval stages. Chironomids are rieyed by larger organisms such as fish and birds; therefore any disruption of their levels may hinder the populations of superior animals (Cattaneo et al., 2009). *C. riparius*has the potential to be used in the study of endocrine effects, as the endocrine systems of insects have been hugely described (Klowden, 2013). As Chironomids possess a life cycle which lasts for approximately 25 days, this time period is adequate to investigate and represent chronic exposures in the habitat as well as a suitable time frame for laboratorial trials. Additionally they possess an adequate size for laboratorial cultures and assays without the need of specialized apparatuses. Chironomids reproduces sexually, thus allowing for a certain degree of genetic variation and providing better insight to the potential response of natural populations. Cultures of chironomids can be easily maintained by feeding organismswith a pre-prepared industrial mixture of fish food, providing an advantage to other test organisms which need additional cultures to provide food (e.g., algal cultures to feed daphnids) (OECD, 2004(a); OECD, 2004(b); Weltje, 2006(b))

Ecotoxicity tests with *C. riparius* present also some drawbacks that should be considered with some caution. When performing life cycle tests, the sex ratio in the swarm may have an influence on the egg rope fertility. As such when extrapolating the decrease in egg rope fertility, it must be determined if it was due to the progenitors' sex ratio, mainly caused by the lower fraction of males, or if it is a real cause-effect from chemical exposure. One of the reasons for the lower ratio of males may derive from their higher sensitivity when compared to females, which in turn is correlated to their higher development rate. As male chironomids emerge earlier than females, this causes a gradual shift in the sex ratio in the breeding cage from 100% males at the beginning of emergence to 100% females at the end of the test. This natural shift may be a hinderer while doing life-cycle test, as a higher desynchronized emergence will lead to infertile egg ropes. (OECD, 2004(a); OECD, 2004(b); Weltje, 2006(b); Gourmelon and Ahtiainen, 2007)

1.7 Aim of the dissertation

This study was undertaken with two major goals:

1. To evaluate toxicity of AgNPs throughout the life cycle of *Chironomus riparius.* This was carried out along with $AgNO_3$ toxicity testing, to try to derive particle-related toxicity.

2. To assess differences between exposure media in ecotoxicity testing,by comparing differences in effects occurring upon two exposures routes: contaminated sediment and spiked water with silver compounds.

To achieve these objectives *C. riparius* bioassays were carried out withseveral life stages, through different methods: acute toxicity in egg masses and first instar larvae; biomarkers in the 4^{th} instar; chronic toxicity at the end and life cycle bioassays; chronic toxiciy was assessed in water column and sediment exposures.

1.8 Relevance of the study

As stated above, it is predicted that Ag release to the environment is increasing, and several Ag forms are expected to occur in aquatic ecosystems. Water and sediments are two important routes of exposure to aquatic organisms. Therefore, *C. riparius* whose life cycle encompasses stages in both the water column and sediment can be a relevant organism to evaluate deleterious effects from stressors present in aquatic systems.

To the best of our knowledge few toxicity studies have been so comprehensive and analysed the water versus sediment toxicity in the different life stages of *C. riparius* and during the course of its life cycle. The majority comprehends studies regarding only the effects found in spiked water thus there is a general lack of studies in contaminated sediments (Lachance et al., 2014).

As AgNPs have different chemical and physical properties than its ionic counterpart, the comparison of both forms is essential to derive an accurate hazard assessment of silver. This comparison in toxicity between different forms can also enlighten on the relevant fractions behind the trigger of toxicity of AgNPs (i.e. ionic, particulate, complexes). NPs fate studies in aquatic systems have highlighted the potential of particles to sink and therefore sediment. Therefore, the use of organisms that will be in direct contact with sediments is crucial to ensure a correct risk assessment and it is vital to assess toxicity to benthic organisms as a first toxicity battery test (Handy et al., 2012).

1.9 Organization of the thesis

The present thesis is structured in four chapters. The second and third chapters are structured as scientific papers.

Chapter 1. General Introduction

The present chapter describes the state of the art of nanoparticles, focusing mainly on silver nanoparticles and silver ions, their sources and properties. The main aims and relevance of the study are also presented.

Chapter 2. Silver nanoparticles' toxicity in different life-cycle stages of the midge *Chironomus riparius*.

Bioassays on the toxicity of silver nanoparticles and silver ions in *Chironomus riparius* when exposed to contaminated aquatic medium. The tests performed evaluated acute toxicity, hatching, growth parameters, adult dry weight and time to emergence.

Chapter 3. Silver nanoparticles' toxicity to *Chironomus riparius* in contaminated sediments.

Evaluation in contaminated sediment with *Chironomus riparius* assessing the effect of silver nanoparticles, with endpoints in growth parameters, sex ratio, enzymatic activity, time to emergence, adult dry weight and fertility rate in two consecutive generations.

Chapter 4. General discussion and conclusion

The last chapter provides an overall review and some final remarks.

1.10 References

ALLAN, J.D. and CASTILLO M.M., 2007. Stream Ecology: Structure and function of running waters Second Edition, Springer, Dordrecht, The Netherlands ISBN 978-1-4020-5582-9.

ALLI S.M.A., 2012. Nanoscience and Nanotechnology: Issues and Applications in Agri-Food, Biomedical, and Environmental Sciences. International Journal of Pharmaceutical Frontier Research IJPFR, 2(1), 61-86.

AITKEN R.J. and TRAN C.L., 2004. Nanoparticles an occupational hygiene review 2004 HSE Books ISBN 0 7176 2908 2.

ARMITAGE P.D., CRANSTON P., PINDER L.C.V., 1995. The Chironomidae: biology and ecology of non-biting midges. Chapman & Hall, London.

ASHARANI P.V., WU Y.L., GONG Z. and VALIYAVEETTI S., 2008. Toxicity of silver nanoparticles in zebrafish models. Nanotechnology, 19(25).

BISWAS P. & WU C.Y., 2005. 2005 Critical review: nanoparticles and the environment. Journal of the Air & Waste Management Association 55,708–746.

BLASER S.A., SCHERINGER M., MACLEOD M. and HUNGERBÜHLER K., 2008. Estimation of cumulative aquatic exposure and risk due to silver: contribution of nano-functionalized plastics and textiles. Science of the Total Environment, 390, 396–409.

BURY N.R. and CHRIS M. WOOD C.M., 1999. Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+ channel. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 277(5), R1385-R1391.

CANFIELD T.J., DWYER F.J., FAIRCHILD J.F., HAVERLAND P.S., INGERSOLL C.I., KEMBLE N.E., MOUNT D.R., LA POINT T.W., BURTON G.A. and SWIFT M.C., 1996. Assessing contamination in Great Lakes sediments using benthic invertebrate communities and the sediment quality triad approach. Journal of Great Lakes Research, 22, 565–583.

CATTANEO A.G., GORNATI R., CHIRIVA-INTERNATI M. and BERNARDINI G., 2009. Ecotoxicology of nanomaterials: the role of invertebrate testing. Invertebrate Survival Journal, 6, 78–97.

CHAPPIE D.J. and BURTON G.A.JR., 1997. Optimization of in situ bioassays with Hyalella azteca and Chironomus tentans. Environmental Toxicology and Chemistry, 16 (3), 559-564.

CHAUDHURI R. G. and PARIA S., 2012. Core/Shell nanoparticles: classes, properties, synthesis mechanisms, characterization, and applications. Chemical Reviews 112, 2373-2433.

CHOI O., DENG K.K., KIM N.J., ROSS L.JR., SURAMPALLI R.Y. and HU Z., 2008. The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth. Water Research, 42(12), 3066-74.

CHOW J.C., WATSON J.G., SAVAGE N., SOLOMON J., CHENG Y., MCMURRY P.H., COREY L.M., BRUCE G.M., PLEUS R.C., BISWAS P. and WU C., 2005. Nanoparticles and the Environment. J. Air & Waste Manage. Assoc., 55, 1411–1417.CHRISTIAN P.C., VON DER KAMMER F., BAALOUSHA M., HOFMANN T., 2008. Nanoparticles: structure, properties, preparation and behaviour in environmental media. Ecotoxicology, 17, 326-343.

CODE OF FEDERAL REGULATIONS, 2010. Office of the Federal Register National Archives and Records U.S. Government Printing Office, Washington, DC C.F.R. 152.15.

COLOMBAN P. and TOURNIÉ A., 2007. On-site Raman identification and dating of ancient/modern stained glasses at the Sainte-Chapelle, Paris, Journal of Cultural Heritage, 8(3), 242-256.

CUERVO A.M., 2004. Autophagy: in sickness and in health. Trends in Cell Biology, 14, 70–7.

D'ALMEIDA G.A., 1989. Desert aerosol: characteristics and effects on climate. M. Leinen and M. Sarnthein (eds.), Paleoclimatology and Paleometeorology: Modern and Past patterns of Atmospheric Transport, 311-338, Kluwer Academic Publishers.

DE LANGE H.J., DE HAAS E.M., MAAS H. and PEETERS E.T.H.M., 2005. Contaminated sediments and bioassay responses of three macroinvertebrates the midge larva *Chironomus riparius* the water louse *Asellus aquaticus* and the mayfly nymph *Ephoron virgo*. Chemosphere, 61, 1700-1709.

DOMINGUES I., GUILHERMINO L., SOARES A.M.V.M. and NOGUEIRA A.J.A., 2007. Assessing dimethoate contamination in temperate and tropical climates: Potential use of biomarkers in bioassays with two chironomid species. Chemosphere, 69(1), 145-154.

DONALDSON K. and STONE V., 2003. Current hypotheses on the mechanisms of toxicity of ultrafine particles. Annali dell'Istituto Superiore di Sanità, 39(3), 405-4110.

DUNN K. and EDWARDS-JONES V., 2004. The role of Acticoat with nanocrystalline silver in the management of burns. Burns, 30(1), S1-9.

EL NEMR A. and ABD-ALLAH A.M.A., 2003. Contamination of polycyclic aromatic hydrocarbons (PAHs) in microlayer and subsurface waters along Alexandria coast, Egypt. Chemosphere, 52, 1711–1716.

EUROPEAN PARLIAMENT AND COUNCIL. Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998 concerning the placing of biocidal products on the market.

EUROPEAN PARLIAMENT AND COUNCIL. Directive 2009/107/EC of the European Parliament and of the Council of 16 September 2009 amending Directive 98/8/EC concerning the placing of biocidal products on the market as regards the extension of certain time periods

EUROPEAN COMISSION, 2011. Recommendations, Comission recommendation of 18 October 2011 on the definition of nanomaterial. Official Journal of the European Union, L 275, 38 – 40.

FAGAN P.J., CALABRESE J.C. and MALONE B., 1991. The chemical nature of Buckminster fullerene (C60) and the characterization of a platinum derivative. Science, 252, 1160 – 1161.

FARIA M.S., RÉ A., MALCATO J., SILVA P.C.L.D., PESTANA J., AGRA A.R., NOGUEIRA A.J.A. and SOARES A.M.V.M., 2006. Biological and functional responses of in situ bioassays with *Chironomus riparius* larvae to assess river water quality and contamination. Science of the total environment, 371(1), 125-137

FELCHER E.M., 2008. The consumer product safety commission and nanotechnology. The Woodrow Wilson International Center for Scholars. Project on Emerging Nanotechnologies. http://www.nanotechproject.org/process/assets/ files/ /8277/pen_submission_cpsc_aug18.pdf (Acessed 31 of May, 2014).

FREITAS R.A., 2005. What is nanomedicine? Nanomedicine 1:2–9 Fu GF, Vary PS, Lin CT (2005) Anatase TiO2 nanocomposites for antimicrobial coatings. Journal of Physical Chemistry, 109, 8889–8898.

FUBINI B. and FENOGLIO I. (2007). Toxic Potential of Mineral Dusts. Mineral Society of America 3(6), 407-414.

GETLER A.O., RHOADS C.P. and WEISS S., 1927. A Contribution to the Pathology of Generalized Argyria with a Discussion of the Fate of Silver in the Human Body. American Journal of Pathology, 3(6): 631– 652.

GOURMELON A. and AHTIAINEN J., 2007. Developing Test Guidelines on invertebrate development and reproduction for the assessment of chemicals, including potential endocrine active substances - The OECD perspective. Ecotoxicology, 16, 161-167.

GOTTSCHALK F., SUN T.Y. and NOWACK B., 2013. Environmental concentrations of engineered nanomaterials: Review of modelling and analytical studies. Environmental Pollution, 181, 287-300.

GROENENDIJK D., POSTMA J.F., KRAAK M.H.S., and ADMIRAAL W., 1998. Seasonal dynamics and larval drift of Chironomus riparius (Diptera) in a metal contaminated lowland river. Aquatic Ecology, 32, 341–351.

HANDY R.D., CORNELIS G., FERNANDES T., TSYUSKO O., DECHO A., SABO-ATTWOOD T., METCALFE C., STEEVENS J.A., KLAINE S.J., KOELMANS A.A. and HORNE N., 2012.

Ecotoxicity test methods for engineered nanomaterials: practical experiences and recommendations from the bench. Environmental Toxicology and Chemistry, 31, 15-31.

HANDY R.D. and, SHAW B.J. 2007. Toxic effects of nanoparticles and nanomaterials: implications for public health, risk assessment and the public perception of nanotechnology. Health Risk Society, 9, 125–144.

HARRISON R.M. and JONES M., 1995. The chemical composition of airborne particles in the UK atmosphere. The Science of the Total Environment, 168, 195-214.

HARRISON P. (ed), 2007. Emerging challenges: nanotechnology and the environment. GEO Year Book 2007. United Nations Environment Programme (UNEP), Nairobi, Kenya, pp 61–68. ISBN 978-92-807-2768-9AVA.

HASSELLÖV M. and KAEGI R.. Analysis and characterization of manufactures nanoparticles in aquatic environments in Environmental and human health impacts of nanotechnology. Lead, JR and Smith E. Blackwell Publishing Ltd. 2009 Chap. 6.

HOLISTER P., Weener J.W., Román C., Harper T., 2003. Nanoparticles Technology White Papers. Científica, 3.

HOUGHTON J., 2005. Global warming. Reports on Progess in Physics, 68, 1343-1403.

IBARRA L.E., TARRES L., BONGIOVANNI S., BARBERO C.A., KOGAN M.J., RIVAROLA V.A., BERTUZZI M.L. and YSLAS E.I., 2015. Assessment of polyaniline nanoparticles toxicity and teratogenicity in aquatic environment using *Rhinella arenarum* model. Ecotoxicology and Environmental Safety, 114, 84-92.

KARNIK B.S., DAVIES S.H., BAUMANN M.J. and MASTEN S.J., 2005. Fabrication of catalytic membranes for the treatment of drinking water using combined ozonation and ultrafiltration. Environmental Science & Technology., 39, 7656–7661.

KIM S.W., BAE D.S., SHIN H. and HONG K.S., 2004. Optical absorption behaviour of platinum core–silica shell nanoparticle layer and its influence on the reflection spectra of a multi-layer coating system in the visible spectrume range. Jounal of Physics: Condensed Matter, 16, 3199–3206.

KIRGIZ T., 1988. A preliminary study on Chironomidae Larvae in Lake Gala. IX. National Biology Congress, Cumhuriyet Univ., Fen Edebiyat Faculty, Biology Department, Sivas, 11 pp.

KLOK C. and KRAAK M.H.S., 2008. Living in highly dynamic polluted river floodplains, do contaminants contribute to population and community effects? Science of the Total Environment, 406, 455-461.

KLOWDEN M.J., 2013. Physiological systems in insects. Academic Press. London UK. ISBN: 978–0-12-415819-1.

KOBASKO N., 2011. An Explanation of Possible Damascus Steel Manufacturing Based on Duration of Transient Nucleate Boiling Process and Prediction of the Future of Controlled Continuous Casting. International Journal of Mechanics, 5(3), 182 – 190.

KÖHLER A.R., SOM C., HELLAND A. and GOTTSCHALK F., 2008. Studying the potential release of carbon nanotubes throughout the application life cycle. Journal of Cleaner Production, 16, 927–37.

KVITEK L., VANICKOVA M., PANACEK A., SOUKUPOVA J., DITTRICH M., VALENTOVA E., PRUCEK R., BANCIROVA M., MILDE D. and ZBORIL R., 2009. Initial Study on the Toxicity of Silver Nanoparticles (NPs) against *Paramecium caudatum.* Journal of Physical Chemistry, C, 113, 4296–4300.

LACHANCE B., HAMZEH M. and SUNAHARA G.I., 2014. Environmental fate and ecotoxicology of nanomaterials in Nanotechnology and human health. Boca Raton: CRC Press, 2014 ISBN 13:978-0-8493-8144-7. Chap. 10, p. 177-201.

LANGMANN B., FOLCH A., HENSCH M. and MATTHIAS V., 2012. Volcanic ash over Europe during the eruption of Eyjafjallajökull on Iceland, April-May 2010. Atmospheric Environment, 48, 1-8.

LARUE C., KHODJA H., HERLIN-BOIME N., BRISSET F., FLANK A.M., FAYARD B., CHAILLOU S. and CARRIÈRE M., 2011. Investigation of titanium dioxide nanoparticles toxicity and uptake by plants. Journal of Physics: Conference Series, 304 (1).

LEAD J.R. and WILKINSON K.J., 2006. Aquatic colloids and nanoparticles: current knowledge and future trends. Environmental Chemistry, 3, 159–171.

LEE S.H. and RICHARDS R.J., 2004. Montserrat volcanic ash induces lymph node granuloma and delayed lung inflammation. Toxicology, 195, 155–165.

LIAU S.Y., READ D.C., PUGH W.J., FURR J.R. and RUSSEL A.D., 1997. Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterial action of silver ions. Letters in Applied Microbiology, 25, 279-283.

LOK C.N., HO C.M., CHEN R., HE Q.Y., YU W.Y., SUN H., TAM P.K., CHIU J.F. and CHE C.M., 2006. Proteomic analysis of the mode of antibacterial action of silver nanoparticles. Journal of Proteome Research, 5(4), 916-24.

LUTHER W. (ed) 2004. Technology analysis, industrial application of nanomaterials – chances and risks. Future technologies division, vdi technologiezentru GmbH, Dusseldorf, Germany. http://www.innovationsbegleitung.de/11.pdf.

MAASRI A., FAYOLLE S., GANDOUIN E., GARNIER R. and FRANQUET E., 2008. Epilithic chironomid larvae and water enrichment: is larval distribution explained by epilithon quantity or quality? Journal of the North American Benthological Society, 27(1), 38–51.

MAFUNÉ F., KOHNO J., TAKEDA Y., KONDOW T. and SAWABE H., 2000. Structure and stability of silver nanoparticles in aqueous solution produced by laser ablation. Journal of Physical Chemistry B, 104, 8333–8337.

MARINKOVIĆ M., DE BRUIJN K., ASSELMAN M., BOGAERT M. JONKER M.J., KRAAK M.H.S. and ADMIRAAL W., 2012. Response of the nonbiting midge *Chironomus riparius* to multigeneration toxicant exposure. Environmental Science & Technology, 46, 12105-12111.

MASCIANGIOLI T. and ZHANG W.X., 2003. Environmental technologies at the nanoscale. Environmental Science & Technology, 37, 103A-108A.

MERRIT R.W. and CUMMINS K.W., 1996. Aquatic Insects of North America. 3rd eds. Kendall / Hunt Publishing Company, Dubuque.

MOORE M.N., LOWE D.M., SOVERCHIA C., HAIGH S.D. and HALES S.G., 1997. Uptake of a noncalorific, edible sucrose polyester oil and olive oil by marine mussels and their influence on uptake and effects of anthracene. Aquatic Toxicology, 39, 307–20.

MUELLER N.C. and NOWACK B., 2008. Exposure Modeling of Engineered Nanoparticles in the Environment. Environmental Science & Technology 42, 4447–4453.

MURR L.E., ESQUIVEL E.V., BANG J.J., DE LA ROSA G. and GARDEA-TORRESDEY J.L., 2004. Chemistry and nanoparticulate compositions of a 10,000 year-old ice core melt water. Water Research, 38, 4282–4296.

NAVARRO E., BAUN A., BEHRA R., HARTMANN N.B., FILSER J., MIAO A.J., QUIGG A., SANTSCHI P.H. and SIGG L., 2008. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. Ecotoxicology, 17, 372-386.

OBERDÖRSTER E., 2004. Manufactured nanomaterials (fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass. Environmental Health Perspectives, 112, 1058–62.

OBERDÖRSTER G., OBERDÖRSTER E. and OBERDÖRSTER J., 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environmental Health Perspectives, 113, 823–839.

OBERNOSTERER I., CATALA P., REINTHALER T., HERNDL G.J. and LEBARON P., 2005. Enhanced heterotrophic activity in the surface microlayer of the Mediterranean Sea. Aquatic Microbial Ecology, 39, 293–302.

OECD, 2001. Guideline 219 - Sediment-water chironomid toxicity test using spiked water. OECD Guidelines for the testing of chemicals.

OECD, 2004a. Test guideline 218 Sediment-Water Chironomid Toxicity Test Using Spiked Sediment. OECD Guidelines for the testing of chemicals.

OECD, 2004b. Test guideline 219 Sediment-Water Chironomid Toxicity Test Using Spiked Water. OECD Guidelines for the testing of chemicals.

PEETERS E.T.H.M., GARDENIERS J.J.P. and KOELMANS A.A., 2000. Contribution of trace metals in structuring in situ macroinvertebrate community composition along a salinity gradient. Environmental Toxicology and Chemistry, 19, 1002–1010.

PEN, The Project on Emerging Nanotechnologies, Consumer Products Inventory (Acessed 31 May 2014) Available on http://www.nanotechproject.org/cpi/.

PIDGEON N., HARTHORN B. and SATTERFIELD T., 2011. Nanotechnology risk perceptions and communication: emerging technologies, emerging challenges. Risk Analysis, 31(11), 1694-1700.

PIXSHARK (Accessed 3 March 2015) http://pixshark.com/chironomidae-midges.html.

PRABHU S. and POULOSE E.K., 2012. Silver nanoparticles: mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects International Nano Letters, 2, 32.

RASMUSSEN J.J., MONBERG R.J., BAATTRUP-PEDERSEN A., CEDERGREEN N., WIBERG-LARSEN P., STROBEL B. and KRONVANG B., 2012. Effects of a triazole fungicide and a pyrethroid insecticide on the decomposition of leaves in the presence or absence of macroinvertebrate shredders. Aquatic Toxicology, 118-119 (0), 54-61.

REID B.J., JONES K.C. and SEMPLE K.T., 2000. Bioavailability of persistent organic pollutants in soils and sediments—a perspective on mechanisms, consequences and assessment. Environmental Pollution. 108, 103–112.

RIETMEIJER F.J.M. and MACKINNON I.D.R., 1997. Bismuth oxide nanoparticles in the stratosphere. Journal of Geophysical Research: Planets, 102, 6621–6627.

SAWOSZ E., BINEK M., GRODZIK M., ZIELINSKA M., SYSA P., SZMIDT M., NIEMIEC T. and CHWALIBOG A., 2007. Influence of hydrocolloidal silver nanoparticles on gastrointestinal microflora and morphology of enterocytes of quails. Archives of Animal Nutrition, 61, 444-451.

SCHULZ J., HOHENBER H., PFUCKER F., GARTNER E., WIII T., PFEIFFER S., WEPT R., WENDEL V., GER-BARTAG H. and WITTEM K.P., 2002. Distribution of sunscreens on skin. Advanced Drug Delivery Reviews Supplement, 1:S157-63.

SCOW K., GOYE M., NELKEN L., PAYNE E., SATERSON K., WALKER P., WOOD M., 1981. An exposure and risk assessment for silver. Technical Report. US Environmental Protection Agency, Washington, DC.

SHAH C.P., Public Health and Preventive Medicine in Canada, 5th ed., W.B. Saunders Canada, 2003.

SMITH C.J., SHAW B.J., HANDY R.D., 2007. Toxicity of single walled carbon nanotubes on rainbow trout, (Oncorhynchus mykiss): respiratory toxicity, organ pathologies, and other physiological effects. Aquatic Toxicology., 82, 94 – 109.

SOARES S., CATIVA I., MOREIRA-SANTOS M., SOARES A.M.V.M. andRIBEIRO R., 2005. A short-term sublethal in situ sediment assay with Chironomus riparius based on postexposure feeding. Archives of Environmental Contamination and Toxicology 49 (2), 163-172.

STYLIOS G.K., GIANNOUDIS P.V., and WAN T., 2005. Applications of nanotechnologies in medical practice. Injury, International Journal of the Care of the Injured 36S, S6—S13.

SUMAN T.Y., RAJASREE S.R.R. and KIRUBAGARAN R., 2015. Evaluation of zinc oxide nanoparticles toxicity on marine algae*chlorella vulgaris* through flow cytometric, cytotoxicity and oxidative stress analysis. Ecotoxicology and Environmental Safety, 113, 23-30.

SWIFT D.L., STRONG J.C., 1996. Nasal Deposition of Ultrafine Aerosols in Human Subjects; Journal of Aerosol Science, 27, 1125-1132.

TAYLOR D.A., 2002. Dust in the wind. Environmental Health Perspectives. Vol. 10, nº2.

TAYLOR L.A., SCHMITT H.H., CARRIER W.D. and NAKAGAWA M. (2005, January). The lunar dust problem: from liability to asset. In 1st Space Exploration Conference: Continuing the Voyage of Discovery. *AIAA* (p. 184).

TESH, S. J. and SCOTT T.B., 2014. Nano-composites for water remediation: a review. Advanced Materials, 26(35), 6056-6068.

TOURINHO P.S., VAN GESTEL C.A.M., LOFTS S., SVENDSEN C., SOARES A.M.V.M and LOUREIRO S., 2012. Metal-based nanoparticles in soil: Fate, behavior, and effects on soil invertebrates. Environmental Toxicology and Chemistry, 31(8), 1679-1692.

TUGULEA A. M., BERUBE D., GIDDINGS M., LEMIEUX F., HNATIW J., PRIEM J. and AVRAMESCU M.L., 2014. Nano-silver in drinking water and drinking water sources: stability and influences on disinfection by-product formation. Environmental Science and Pollution Research International, 21(20), 11823-11831.

TUPLING R. and GREEN H., 2002. Silver ions induce Ca^{2+} release from the SR in vitro by acting on the Ca^{2+} release channel and the Ca^{2+} pump. Journal of Applied Physiology, 92(4), 1603-1610.

VAN BERKEL K.Y., PIEKARSKI A.M., KIERSTEAD P.H., PRESSLY E.D., RAY P.C., and HAWKER C.J., 2009. A Simple Route to Multimodal Composite Nanoparticles Macromolecules, 42, 1425-1427.

VERMA H.C., UPADHYAY C., TRIPATHI A., TRIPATHI R.P. and BHANDARI N., 2002. Thermal decomposition pattern and particle size estimation of iron minerals associated with the cretaceous-tertiary boundary at Gubbio. Meteoritics & Planetary Science, 37, 901–909.

VERT M., DOI Y., HELLWICH K., HESS M., HODGE P., KUBISA P., RINAUDO M., and SCHUÉ F., 2012. Terminology for biorelated polymers and applications (IUPAC Recommendations 2012) Pure and Applied Chemistry, 84(2): 377–410.

VIARENGO A. and NOTT JA., 1993. Mechanisms of heavy-metal cation homeostasis in marine invertebrates. Comparative Biochemistry and Physiology, 104:355–72.

VOGT C., HEß M., NOWAK C., DIOGO J., OEHLMANN J. and OETKEN M., 2010. Effects of cadmium on life-cycle parameters in a multi-generation study with Chironomus riparius following a pre-exposure of populations to two different tributyltin concentrations for several generations. Ecotoxicology, 19(7), 1174-1182.

VOGT C., PUPP A., NOWAK C., JAGODZINSKI L.S., BAUMMANN J., JOST D., OETKEN M. and OEHLMANN J., 2007. Interaction between genetic diversity and temperature stress on life-cycle parameters and genetic variability in midge *Chironomus riparius* populations. Climate Research, 33, 207-214.

WELTJE L., DORGERLOH M., PFEIFLE V., BELZ D., TÄNZLER V. and REINHARD D., 2006b. Development of a life-cycle test with the non-biting midge Chironomus riparius. In: Abstract book SETAC-GLB. Landau, Germany.

WIGGINTON N.S., HAUS K.L., HOCHELLA M.F., 2007. Aquatic environmental nanoparticles. Journal of Environmental Monitoring, 9, 1306–1316.

WHO, WORLD HEALTH ORGANIZATION. World Health Report 2002. Geneva, Switzerland: World Health Organization; 2002.

WU Y.L., TOK A.I.Y., BOEY F.Y.C., ZENG X.T. and ZHANG X.H., 2007. Surface modification of ZnO nanocrystals. Applied Surface Science, 253, 5473 – 5479.

YAH C.S., SIMATE G.S., and IYUKE S.E., 2012.Nanoparticles toxicity and their routes of exposures. Pakistan Journal of Pharmaceutical Sciences, 25(2), 477-491.

YAMADA T., UEDA M., SEMP M., KONDO A., TANIZAWA K. and KURODA S., 2004. Novel tissue and cell type-specificic gene/drug delivery system using surface engineered Hepatites B virus nano-particles. Current Drug Targets-Infectious Disorders, 4(2), 163-167.

ZEYONS O., THILL A., CHAUVAT F., MENGUY N., CASSIER-CHAUVAT C., ORÉAR C., DARASPE J., AUFFAN M., ROSE J. and SPALLA O., 2009. Direct and indirect CeO_2 nanoparticles toxicity for*Escherichia coli* and *Synechocystis.* Nanotoxicology, 3(4), 284-295.

ZHAO C.M and WANG W.X., 2012. Importance of surface coatings and soluble silver in silver nanoparticles toxicity to *Daphnia magna.* Nanotoxicology, 6(4), 361 – 370.

Chapter 2

Silver nanoparticles' toxicity in different life-cycle stages of the midge *Chironomus riparius*

2. Silver nanoparticles' toxicity in different life-cycle stages of the midge Chironomus riparius

2.1 Abstract

Keywords: Silver nanoparticles (AgNPs), *Chironomus riparius*, water, toxicity

This study was focused on the assessment of water toxicity of silver nanoparticles (AgNPs) to the midge *Chironomus riparius*. To evaluate the toxicity of silver compounds in chironomids several endpoints were chosen: hatching, survival in different stages, larval growth and emergence. Hatching was significantly affected in both exposure to AgNPs and $AgNO_3$, with EC_{50}s values of 3882.27 $\mu g.L^{-1}$ and 77.68 $\mu g.L^{-1}$, respectively. Toxicity was induced in midges in the 48 hour toxicity bioassays performed. Mortality was reported, with a LC_{50} for AgNPs of 1026.94 $\mu g.L^{-1}$ and of 23.37 $\mu g.L^{-1}$ for $AgNO_3$. For AgNPs no effects were perceivable in the growth rate of both head and body at ten days of exposure, nevertheless at 28 days there was a delay in the mean time of emergence, with an EC_{50} value of 1289.86 $\mu g.L^{-1}$. An emergence decrease of the number of imagoes was also observed for AgNPs with an EC_{50} value of 843.71 $\mu g.L^{-1}$. These parameters were unresponsive in the concentrations tested in $AgNO_3$. Egg masses showed high resistance to Ag exposure in contrast the first larval stage, which was the most sensitive stage to AgNPs. The lack of toxicity during the first 10 days may indicate a high resistant stage followed by a vulnerable stage during metamorphosis.

2.2 Introduction

Aquatic ecosystems can act as a rinser and a final sink to several compounds regardless of their natural or anthropogenic nature (Lagrana et al., 2010). Nanoparticles (NPs) are one of the emergent chemicals that are of more concern nowadays, due to their increase in production and usage. Silver NPs are among the most used and it has been estimated that between 25 to 40% of the silver (Ag) released to the environment enters amidst the aquatic compartment (Scow et al., 1981). Furthermore, it was also predicted that by 2010, 15% of total silver present in waste water in the European Union would come from biocidal materials (Benn and Westerhoff, 2008) and the market share of these products would further proliferate in the succeeding years (HeiQ, 2006). These estimates have been confirmed in a posterior survey (Burkhardt et al., 2011).

Biocidal silver products can act through different mechanism, being one of the most common way for the long term release of silver ions. This occurs when metallic silver is oxidized due to contact with water (Kumar et al., 2005 and Dobias and Bernier-Latmani, 2013). So far studies regarding the mechanisms by which AgNPs and silver ions derive their toxicity, have shown that different pathways are at play for the two forms. When present in the natural media silver ions act mainly through the depletion of the cellular energy system through competition with Na^+ in the membrane sodium channels and adenosine triphosphate (ATP) dependent transport. Silver ions also affect ATP synthesis and DNA transcriptions and are capable of blocking the cycles of phosphorus, sulphur and nitrogen in nitrifying bacteria (Bianchini and Wood, 2003). On the other hand, several studies, both *in vivo* and *in vitro*, have proven that NPs can enter cells through diffusion, pinocytosis and phagocytosis (Luoma, 2008 and Park et al., 2010). They act mainly through the generation of reactive oxygen species and are linked with damages in cell by processes of protein alteration, DNA disruption, lipid peroxidation, interference with signalling functions and gene transcription. (Brown et al., 2004 and Oberdörster et al., 2005).

Further from these mechanisms of toxicity, NPs are also capable of entering organisms' cells and interact with subcellular structures (Kloepfer et al., 2005 and Morones

et al., 2005) possibly by passive uptake, as metallic NPs do not possess specific receptors on their exterior layer. This relation might be instigated by artificial tensions effects, electrostatic charges, steric interactions, or Van der Waals forces (Peters et al., 2006 and Xia et al., 2006). Therefore the potential toxicity and ability to catalyse oxidative products will rely on the magnitude of exposure, nanoparticle properties (chemistry, size, and shape) (Pal et al., 2007), particles internalization in living tissues and their final target, which will lead to different interactions with the cells (Edmond, 2014).

To assess if NPs can induce detrimental effects to the aquatic ecosystem, several approaches can be carried out in order to accurately assess effects. One is to consider time as an important endpoint and straightly related to a complete life cycle of organisms. Therefore choosing different life stages of an organism can be an approach that will elucidate more about effects and specifically to derive which are the most sensitive timeframes of the organisms' life-cycle. Bioassays with chironomids can be implemented in this way, as the most extended period of their life cycle (eggs and larval stage) is spent in contact with the overlaying aquatic medium, and thus continuously exposed to contaminants (Lagrana et al., 2011). Bioassays generally focus on toxicity in the sediment dwelling instars, this approach may misjudge the sensitivity of the entire species (Gauss et al., 1985). Studies have shown that other effects derived from contamination exposure can be measured in several stages of Chironomids, particularly in larval growth, development time and the amount of emerged adults (Azevedo-Pereira et al., 2010 and Azevedo-Pereira et al., 2011).

Therefore, this study aimed at characterizing the potential effects of AgNPs to the midge *C. riparius*, taking advantage of their full life cycle. For that, exposures were run with eggs and first instar larvae, with different exposure times. In addition, by comparing AgNPs with $AgNO_3$ exposures, discrimination between Ag forms was also attempted.

2.3 Materials and Methods

2.3.1 Test Organism

All the organisms used in the experiments were raised at the University of Aveiro's in-house laboratory culture of *C. riparius*. The culture was maintained in aquaria containing quartz sand overlaid with ASTM hard water, at 20 ± 2 °C, 16: 8 h light: dark photoperiod, with an intensity of approximately 1000 lux. These conditions were also applied and maintained throughout the experimental procedures. The culture and larvae used in the experiments were fed with TetraMin® (Tetrawerke, Germany) in a ratio of 1g of TetraMin® per 20 mL of water, three times per week (OECD, 2011).

2.3.2 Test Substances

Chironomids were exposed to two silver compounds: $AgNO_3$, supplied bySigma-Aldrich (St. Louis, MO), with 99% purity (CAS 7761-88-8) and an ultrapure water dispersion of AgNPs, with an initial concentration of 1000 ppm, supplied by AMEPOX Enterprise). Silver nanoparticles in suspension had an initial size between 3 and 8 nm, with an artificial alkane protection layer and a zeta potential of 33mV (NanoSilver Suspension AX 06Hx, 2015). Both reagents were kept at room temperature and in total darkness. The test vessels used in the experiments were of constituted of chemical inert glass, as standard practice in our laboratory. AgNPs characterization in the test medium used in the current experiment were previously characterized by Ribeiro et al. (2014), where the diameter of AgNPs, aggregation experiments on test-media and TEM imaging were performed. Z-average hydrodynamic diameter of the AgNPs were measured using a Malvern Zetasizer and TEM imaging, experiments were carried out on a JEOL 2010 analytical TEM. (Ribeiro et al., 2014)

The artificial ASTM hard water was used as exposure media (ASTM, 2010) and when a sediment complement was needed (e.g. chronic test) artificial sediment was prepared by mixing 75% of quartz sand, 20% of kaolin clay (Sigma) and 5% of α-cellulose (Sigma), adjusted to a pH of 6.25 with $CaCO_3$. The sediment in the beakers created a layer with 1.5 cm in height, providing every larva with an area of over 2.50 cm^2 (OECD, 2004). The AgNPs test suspensions used in each bioassay were prepared by dilution of the initial dispersion in ASTM, to the desired concentrations. The $AgNO_3$ test solutions were prepared by diluting a 50 mg $Ag.L^{-1}$ pre-prepared solution, by the same method

2.3.3 Egg Rope tests

The test design was adapted from (OECD, 2010). The experiment set up included five concentrations for AgNO3 and six for AgNPs, both with the correspondent negative control. Each concentration had five replicates, with one egg rope each. The egg ropes were divided with the aid of a scalpel, in order to provide per replicate a similar number of eggs, which were placed in 50 mL Petri dishes. An extended period of 6 days was used to assess potential delays in hatching time (OECD, 2010). The concentrations used in the exposures were based on range finding tests performed earlier (data not shown), and for AgNPs, concentrations used were 0.25, 0.5, 1, 2 and 4 mg $Ag.L.^{-1}$ and 46.88, 93.75, 187.5, 375, 750 µg $Ag.L^{-1}$ for $AgNO_3$. Hatching success was calculated using the following equation:

$$\text{Hatching success} = \left(\frac{\text{Hatched eggs}}{\text{Total number of eggs}}\right) \times 100$$

2.3.4 Acute tests

Chironomidae larvae of the first instar were exposed to AgNPs and $AgNO_3$ during 48 hours. The test was performed with chironomids from three egg ropes, laid in the same day; hatched chironomids were selected randomly and distributed through the replicates. Larvae were fed only and immediately after hatching. Exposures were run in 50ml petri dishes, covered with a glass lid in order to prevent water evaporation. Larvae immobilization at 48 hours was assessed as an endpoint to indicate mortality (already established or soon to occur), (OECD, 2011).

Five concentrations of AgNPs and six concentrations for $AgNO_3$ exposure were used in the experimental trials, plus a negative control, with five replicates per concentration; in each petri dish five larvae were used. All tested concentrations were obtained from a stock solution of $AgNO_3$ (200 $\mu g.L^{-1}$) and from the emulsion provided by Amepox diluted in ASTM (1000 ppm). The concentrations used in the exposures were based on range finding tests performed earlier (data not shown), and for AgNPs concentrations used were 0.5, 1, 2, 4 and 8 mg $Ag.L.^{-1}$ and 5, 10, 20, 40, 80, 160 µg $Ag.L^{-1}$ for $AgNO_3$.

2.3.5 Chronic tests

A chronic experiments was performed, with two different sampling times 10 days and 28 days. Experiments were carried out in 200mL glass beakers (7 replicates per treatment) with nine larvae (first stage, two days old) per replicate (OECD, 2004). Each replicate contained 50 grams of artificial sediment and 150mL of ASTM and aeration was provided several days prior to the start of the experiment. Larvae were fed every other day with a TetraMin® ratio of 0.5 mg/larvae/day (Pestana, 2009).

Individuals with less than 48 hours were used and the water spiking occurred 24 hours after transferring the test organisms to the vials. The aeration was reestablished 24

hours after spiking. The concentrations used were based on amounts found to be leached to water in the literature (Benn and Westerhoff, 2008 and Lorenz et al., 2012) and in the LC_{50} values found in the acute tests. Concentrations used were also based on previous results obtained in preliminary tests (data not shown) and were of 200, 400, 800, 1600, 3200 µg $Ag.L^{-1}$ for AgNPs and 10, 30, 50, 70, 90 µg $Ag.L^{-1}$ for $AgNO_3$. Higher concentrations were tested in the preliminary trials and showed to induce high mortality rates. Two sampling times were recorded: 10 days and 28 days. An additional number of replicates was sacrificed at 10 days to calculate growth rates and larval survival. Growth rate for both body and head were calculated through the following equation adapted from Leal et al. (2012) where the mean size can be related to the total body length or to the head width:

$$\text{Growth rate} = \frac{(\text{mean final size}) - (\text{mean initial size})}{10 \text{ days}}$$

To achieve that, each organism was preserved in ethanol (70%, and head width and total body length were measured (Faria et al., 2006), using a stereo microscope (MS5, Leica Microsystems, Houston, USA) fitted with a calibrated eye-piece micrometer. To obtain a mean value for the larvae initial size, an additional set of organisms from the same batch were measured at the start of the experiment. The remaining replicates were allowed to run until the end of 28 days, larval emergence was recorded daily and imagoes were sexed and conserved in ethanol (70%) and dried at room temperature thereafter. Their individual dry weight was measured within a microbalance and was used as an additional measurement in this experiment.

2.3.6 Statistical Analysis

To perform the statistical analysis of data, comparisons between the controls and Ag concentrations were carried out by means of a One Way Analysis of Variance. Each and

every time data were not normally distributed, data transformations were carried out; whenever these did not correct for normality, Kruskal-Wallis' One Way Analysis of Variance on Ranks were executed. Whenever significant differences were discernible Holm-Sidak's or Dunn's methods were implemented to distinguish statistical differences amongst groups (SPSS 2008). To calculate the EC_{50}s, nonlinear regressions with sigmoidal functions were carried out, of which the one that presented the best fit-curve was chosen.

2.4 Results

Regarding the physical parameters, no significant changes were observed in pH, oxygenation and conductivity in any of the tests that were completed. In all experiments the values obtained were within the range of limits of the chironomid toxicity test guideline: dissolved oxygen content >60% air saturation value, pH between 6 to 9 and water temperature did not fluctuated by more than 1 ºC (OECD, 2004).

2.4.1 Characterization of the AgNPs

The characterization of the AgNPs in the test media used in this study were previously performed by Ribeiro et al. (2014), which reported stable hydrodynamic diameters of 127–132±4 nm for AgNPs during 2 weeks. In ASTM, the zeta-average diameters of particles agglomerates at day zero was approximately 80 nm, after 24hthe zeta average increased to approximately 200 nm and after 3 days agglomerates reached 350 nm. TEM measurements reported individual particles with 7.5 ± 1.7 nm in diameter. A large number of paraffin covered aggregates of approximately 30–100 nm diameter were also observed. The EDX from the metallic particles showed that there was extremely little sulphur related to the Ag particles (Ribeiro et al., 2014).

2.4.2 Egg rope tests

Hatching started to occur three days after the beginning of testing, and it was discernibly affected at the end of 6 days, showing a clear a dose response reaction for both Ag forms.

For AgNPs it was found a NOEC of 2500 µg.L^{-1}, and a LOEC of 5000 µg.L^{-1}, with an EC_{50} derived of 3882.27 ± 491.22 µg Ag.L^{-1}(One Way ANOVA $F_{5, 24}$ = 74.75, $p<0.05$, Holm-Sidak's method, $p<0.05$; fig. 2.1., table 2.1.).

On the other hand $AgNO_3$ concentration had a NOEC lower than 46.88 µg Ag.L^{-1} with all treatments showing significant differences from the control (fig. 2.2.). The EC_{50} of 77.68 ± 6.07 µg.L^{-1} was therefore calculated for $AgNO_3$ (table 2.1.) (One Way ANOVA $F_{5, 24}$ = 35.30 $p<0.05$, Holm-Sidak's method, $p<0.05$).

Table 2.12. EC_{50}, NOEC and LOEC (as µg Ag.L^{-1}) values obtained from the exposure of egg ropes of *Chironomus riparius* to AgNPs and $AgNO_3$ in ASTM hard water for 6 days.

	EC_{50} value	**Standard error**	**r^2**	**NOEC**	**LOEC**
AgNPs	3,822.87	491.22	0.82	2,500	5,000
$AgNO_3$	77.68	6.07	0.93	<46.88	46.88

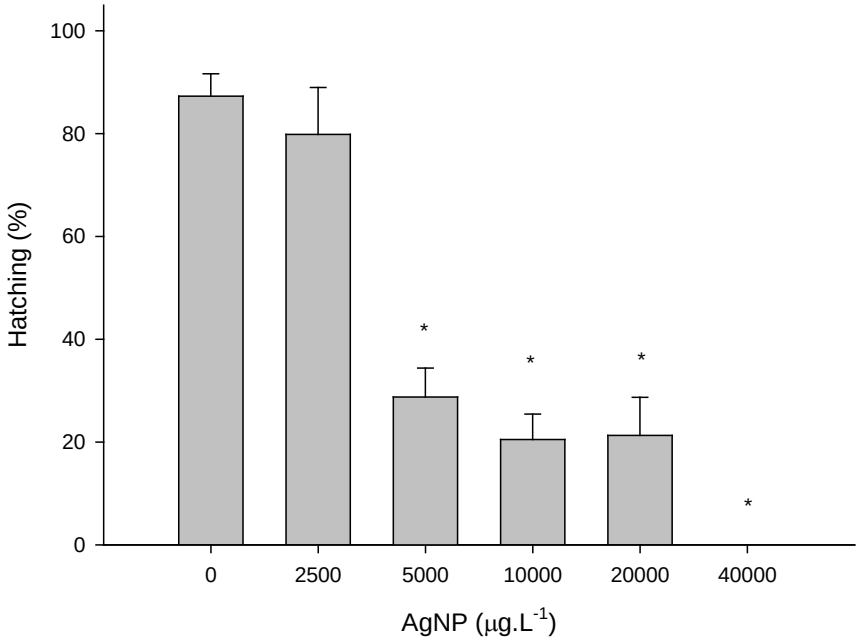

Fig. 2.1. Hatching success (% individuals that spawned) of *Chironomus riparius* egg ropes when exposed to AgNPs in ASTM hard water for 6 days. Data is expressed as mean values and standard error [(*) denotes statistical significant differences (p<0.05 Holm-Sidak's method)].

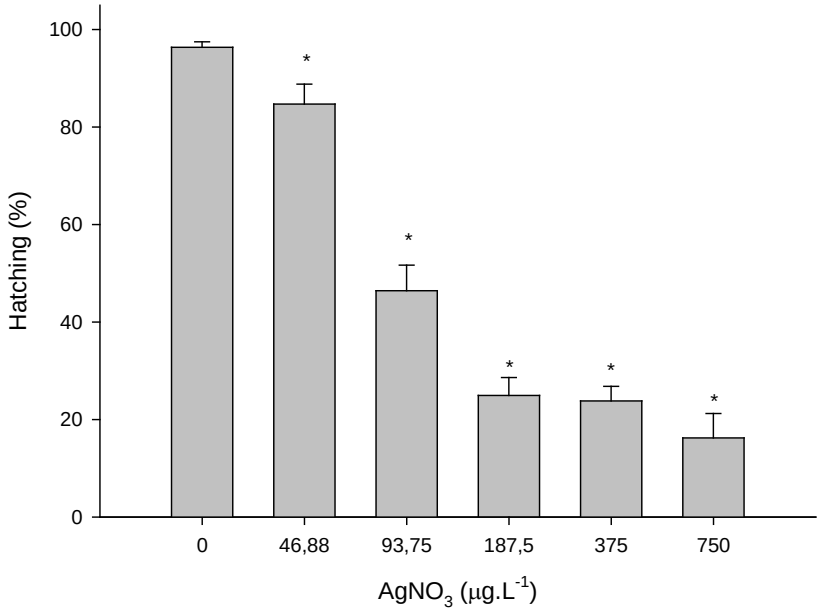

Fig. 2.2. Hatching success (% individuals that spawned) of *Chironomus riparius* egg ropes when exposed to $AgNO_3$ in ASTM hard water for 6 days. Data is expressed as mean values and standard error [(*) denotes statistical significant differences (p<0.05 Holm-Sidak's method)].

2.4.3 Acute tests.

Toxicity was assessed at the end of 48 hours, and a NOEC and LOEC values were obtained for AgNPs of 1000 $\mu g.L^{-1}$ and 2000 $\mu g.L^{-1}$, respectively (fig. 2.3.). In the highest exposure concentration tested there was no live larvae at the terminus of the test; an LC_{50} of 1026.94 ± 117.07 $\mu g.L^{-1}$ was derived (table 2.2) (Kruskal-Wallis ANOVA on Ranks, H=26.56, df= 5, $p<0.05$).

Likewise for $AgNO_3$ an effect-concentration was perceptible with the highest three treatments having significant differences from control, and 160 $\mu g.L^{-1}$ showing to induce a complete mortality at the end of the test (Fig 2.4.). A NOEC of 10 $\mu g.L^{-1}$ and a LOEC of 20 $\mu g.L^{-1}$ were obtained and the LC_{50} of 23.37 ± 1.58 $\mu g.L^{-1}$ was calculated for $AgNO_3$ (table 2.2.) (Kruskal-Wallis ANOVA on Ranks, H=33.00, df= 6, $p<0.05$).

Table 2.2. LC_{50}, NOEC and LOEC (as $\mu g.L^{-1}$) values obtained from the 48 hours' exposure of *Chironomus riparius* to AgNPs and $AgNO_3$ in ASTM hard water.

	LC_{50} value	**Standard error**	**r^2**	**NOEC**	**LOEC**
AgNPs	1026.94	117.07	0.92	500	1000
$AgNO_3$	23.37	1.58	0.96	10	20

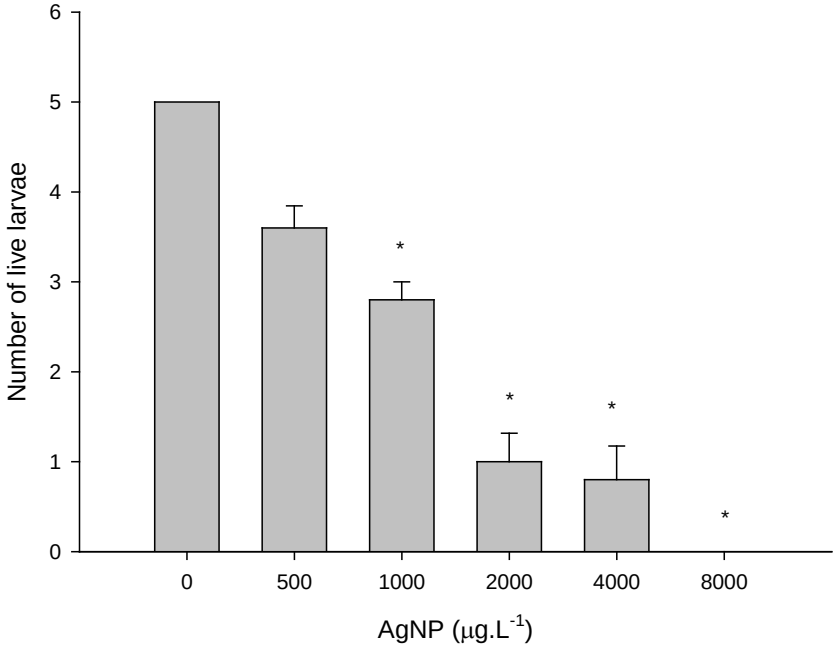

Fig. 2.3. Number of individuals of *Chironomus riparius* which survived upon a 48h exposure to AgNPs in ASTM hard water. Data is expressed as mean values and standard error [(*) denotes statistical significant differences ($p<0.05$ Dunn's method)].

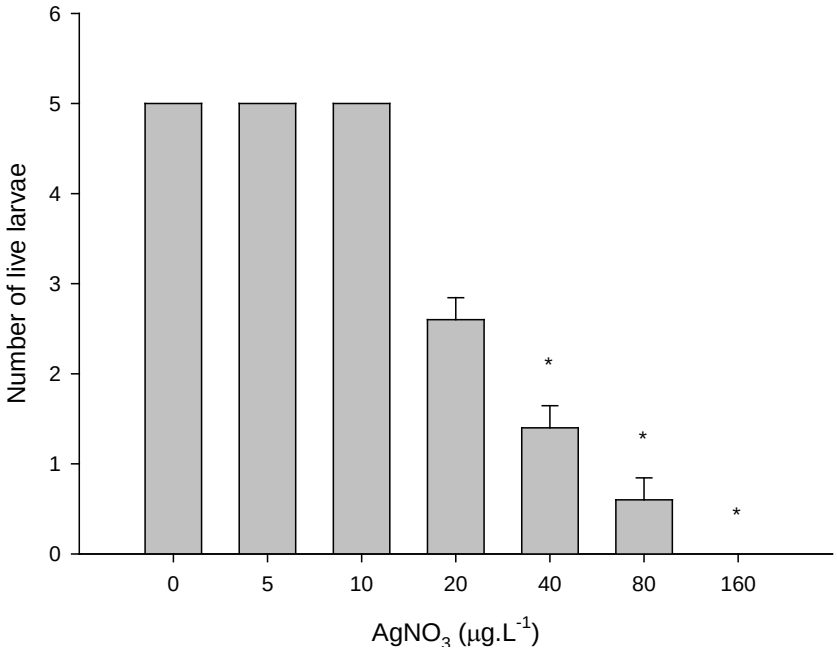

Fig. 2.4. Number of individuals of *Chironomus riparius* which survived upon a 48h when exposed to $AgNO_3$. Data is expressed as mean values and standard error [(*) denotes statistical significant differences ($p<0.05$ Dunn's method)].

2.4.4 Chronic test

After 10 days and for both exposures (AgNPs and $AgNO_3$) there were no statistical differences regarding survival, body growth rate and head growth rate (data not shown). Although some mortality was observed with increasing concentrations of AgNPs, no significance was obtained when tested against the negative control (Fig. 2.5). Growth rate of both body and head did not show any distinct effect pattern (Fig. 2.6).

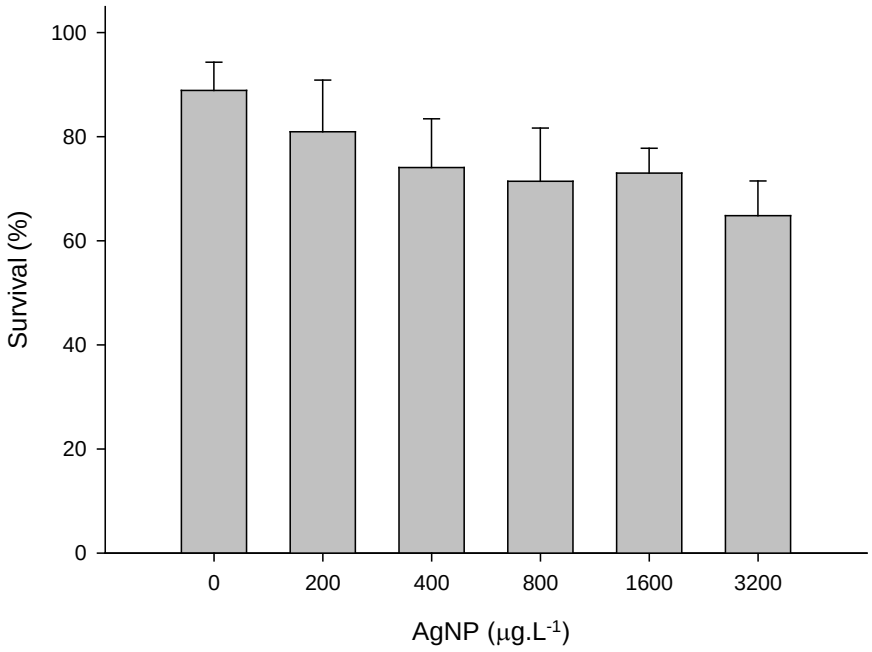

Fig. 2.5. Survival of *Chironomus riparius* upon a 10 day exposure to AgNPs in ASTM hard water. Data is expressed as mean values and standard error.

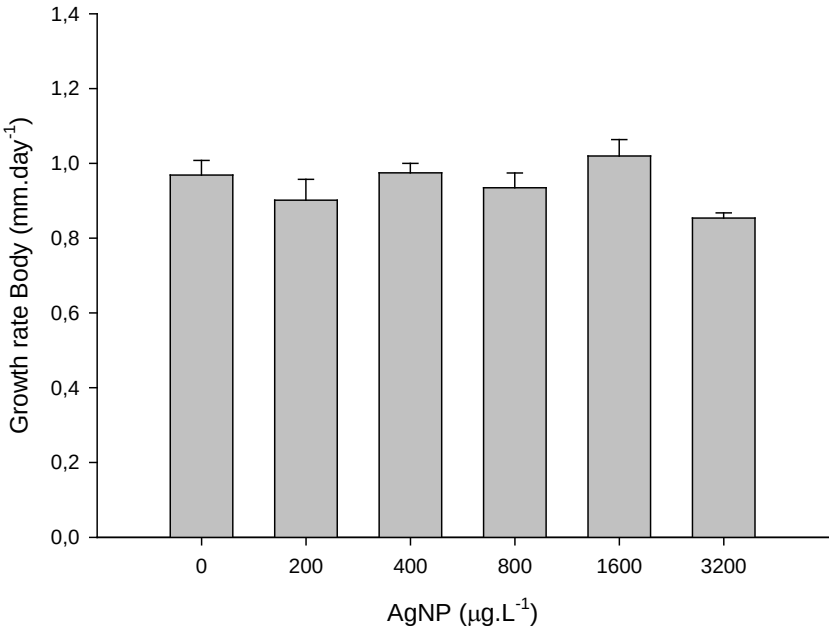

Fig 2.6. Mean growth rate of larval body of *Chironomus riparius* expressed as mm per day, when exposed to AgNPs during 10 days in ASTM hard water. Data is expressed as mean values and standard error.

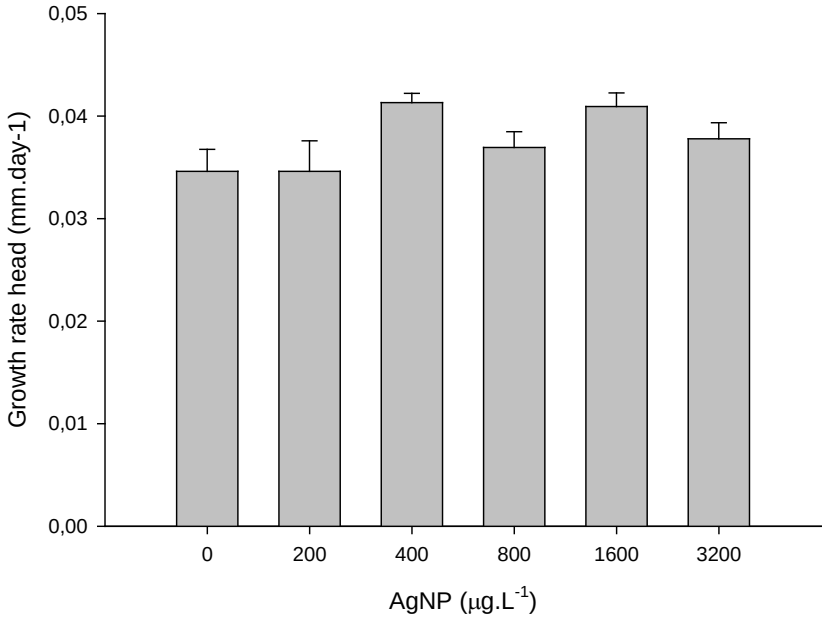

Fig. 2.7. Mean growth rate of larval head of Chironomus riparius expressed as mm per day, when exposed to AgNPs during 10 days in ASTM hard water. Data is expressed as mean values and standard error.

At the end of 28 days for AgNPs exposure emergence success was assessed and a clear dose response curve was observed (fig. 2.8.). The time to emergence values for control were over 80%, with a significant effect observed at 3200 $\mu g.L^{-1}$ below 40% of the total organism present at the beginning of the trials. A NOEC of 400 $\mu g.L^{-1}$, and a LOEC of 800 $\mu g.L^{-1}$ were derived, along with an EC_{50} value of 843.71 ± 170.30 $\mu g.L^{-1}$ (table 2.3.) (Kruskal-Wallis ANOVA on Ranks, H=29.46, df=5, $p<0.05$).

The mean time to emergence showed a clear delay at the highest concentrations with delays of 3 days in comparison with control in average (fig. 2.9.), whilst for 3200 $\mu g.L^{-1}$ was close to day 25 of the experiment whilst the mean day for control was near day 22. The derived NOEC was 800 $\mu g.L^{-1}$, and LOEC of 1600 $\mu g.L^{-1}$, (One Way ANOVA $F_{5, 34} = 8.93$, $p<0.05$, Holm-Sidak's method, $p<0.05$).

Mean weight for male and female chironomids remained relatively constant in all treatments without any statistical significant differences (fig. 2.10.), for male weight (One way ANOVA, $F_{5, 32}=2.46$; $p>0.05$), and female adult weight (One way ANOVA, $F_{5, 36}=0.83$; $p>0.05$). The males' weight was within the range of 0.4 - 0.6 mg per midge which was lower than the females' weight within the range of 1.1 - 1.3 mg per midge. Regarding $AgNO_3$ exposures, 28 day emergence tests revealed no statistical differences up to 90 $\mu g.L^{-1}$ in all three endpoints considered (adult emergence(One way ANOVA, $F_{5, 36}=2.74$; $p>0.05$), mean day of emergence (One way ANOVA, $F_{5, 36}=2.23$; $p>0.05$, male adult weight(One way ANOVA, F5, 36=1.38; $p>0.05$ and female adult weight (One way ANOVA, $F_{5, 35}=0.20$; $p>0.05$) data not shown.

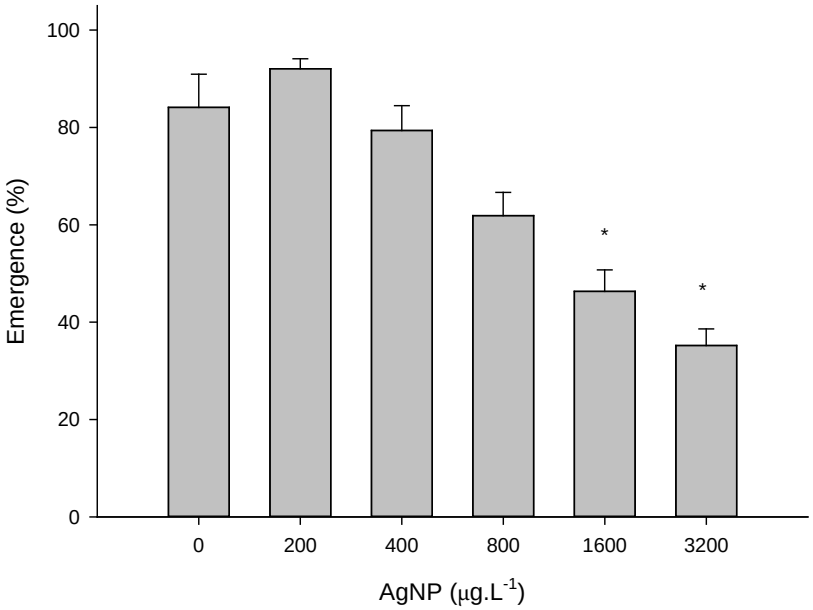

Fig. 2.8. Emergence success of individuals of *Chironomus riparius* that reached adulthood when exposed to AgNPs during 28 days in ASTM hard water. Data is expressed as mean values and standard error [(*) denotes statistical significant differences ($p<0.05$ Dunn's method)].

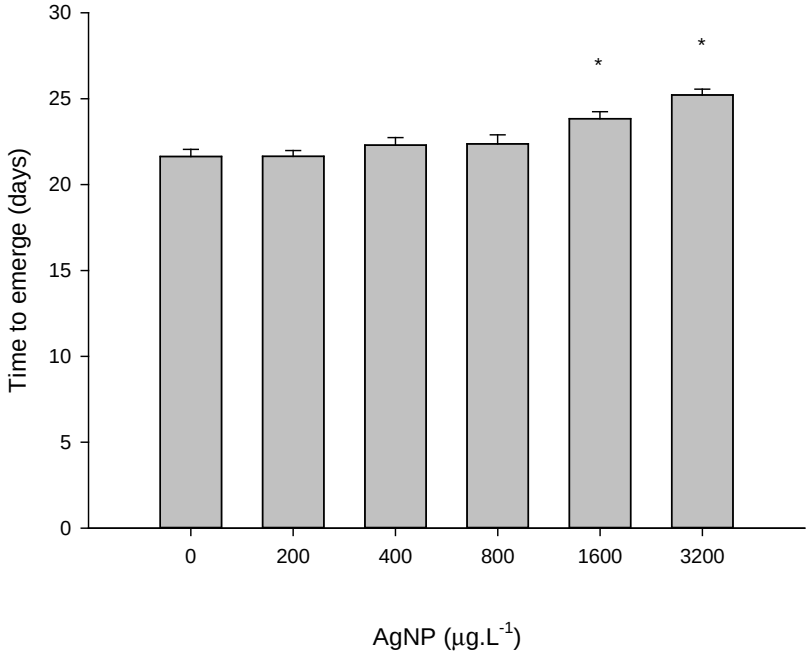

Fig. 2.9. Time to emerge of individuals of *Chironomus riparius* when exposed to AgNPs during 28 days in ASTM hard water. Data is expressed as mean values and standard error [(*) denotes statistical significant differences ($p<0.05$ Holm-Sidak's method)].

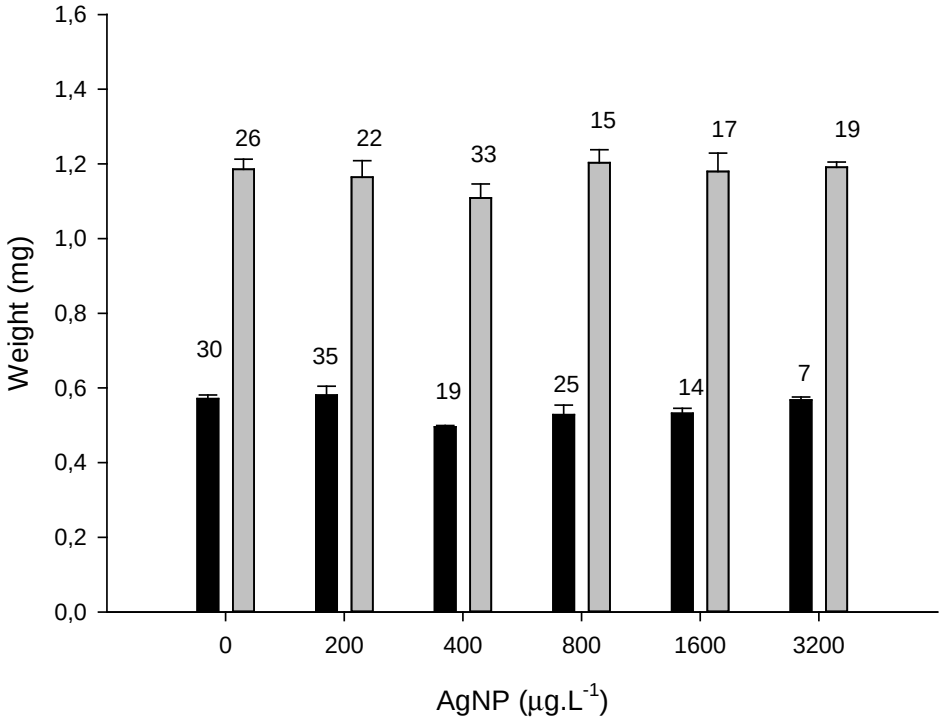

Fig. 2.10. Mean weight of male and female midges of *Chironomus riparius* when exposed to AgNPs during 28 days in ASTM hard water. Data is expressed as mean values and standard error. Black bars are for male weight data and grey bars refer to female weight data.

Table 2.3. EC_{50}, NOEC and LOEC (as $\mu g.L^{-1}$) values obtained from 28 days' exposure of *Chironomus riparius* to AgNPs in ASTM hard water.

Parameter	EC_{50} value	Standard error	r^2	NOEC	LOEC
Emergence	843.71	170.30	0.74	800.00	1600.00

2.5 Discussion

Hatchability, growth, and emergence are known to be affected by several factors such as high temperatures (Frouz et al., 2002), differences in photoperiod (Gong et al., 2002), low pH (Rousch et al., 1997) or food availability (Waissi-Leinonen et al., 2012). These parameters of potential variation, were not at play in this study, as temperatures, photoperiod, pH and food provided were considered optimum and stable throughout the assays, and thus the observable effects must be related exclusively to the toxicants exposures.

The egg toxicity results obtained were in line with previous studies which showed that egg masses from *Chironomus decorus* had a higher resistance to copper exposure than other life stages (Kosalwat and Knight, 1987). The eggs fecundity (e.g. success of hatching) of *C. riparius* was significantly affected by the two Ag forms used; nevertheless AgNPs showed to induce less toxicity than $AgNO_3$, with EC_{50} values showing that $AgNO_3$ is approximately 50 times more toxic than AgNPs. These findings were somehow contradictory with the ones from García-Alonso *et al.* (2014), where effects on fertilized eggs was only observed upon AgNPs exposure. Although using another insect species and only a 4-hour exposure period, it has been reported that egg viability decreases in TiO_2 NPs exposure to *Drosophila melanogaster* but not with AgNPs (20 nm) and a silver nanomaterial (3 µm) (Philbrook et al., 2011). Surprisingly, Gorth *et al.* (2011) using also *D. melanogaster*, found that higher toxicity was observed for eggs hatching when exposed during 12 hours to non-nanometer Ag materials (500 – 1200 nm) when compared to AgNPs with 20-30 nm. Nevertheless AgNPs assessed in these studies had different sizes than the AgNPs used in our study (3-8 nm), and also of major importance, the time for exposure was different, being in the majority of studies of some hours. The greater timespan of exposure in this study could have allowed a greater penetration of AgNPs through the gelatinous matrix of eggs or due to the higher release of ions from the NPs, that is also time dependent.

Midge larvae have been been previously shown to be sensitive to several contaminants such as metals (Rousch et al., 1997). In the performed trials the LC_{50} values

showed that $AgNO_3$ was approximately 44 times more toxic than AgNPs. There was an increase in sensitivity from the egg rope to the first larval stage in *Chironomus riparius'* life cycle. As larvae are waterborne in the initial days of the life cycle, any contaminant in the water column can potentially affect them, leading to the disruption of the life cycle (Gauss et al., 1985; Nebeker et al., 1984; Pascoe et al., 1989; Williams et al., 1986). Therefore immobilization in the first larval instar can be a sensitive and relevant parameter to assess toxicity in *C. riparius* (Vogt et al., 2007a). Indeed in our study first instar midge larvae were more sensitive than the eggs, with $AgNO_3$ being always more toxic than AgNPs. The harmfulness in this life stage can be potentiated by ingestion of nanoparticles and inability for the larvae to move in the higher doses as it occurred to studies with *Daphnia magna* (Asghari et al., 2012).

In the later stages of the chironomids life cycle, growth was reported as a sensitive endpoint, which is impaired by exposure to insecticides (Azevedo-Pereira et al., 2011), mercury (Azevedo-Pereira et al., 2010) and cadmium (Postma et al., 1995). In addition, the 3rd and 4th instar have been reported to accumulate the most metals (Gillis and Wood, 2008). Although effects on growth have been reported in chironomids when exposed to contaminants under laboratorial trials, in our study growth rates were unresponsive.

In oxygen rich conditions, as in our experiment, thermodynamics postulates that AgNPs are not at equilibrium and will not remain in the water column as it will suffer processes such as aggregation (Liu and Hurt, 2010). As tested by Ribeiro et al. (2014), which used the same AgNPs and test media, these AgNPs did not undergo sulphidisation, but nevertheless had the tendency to agglomerate, noticeable after 3 days in ASTM hard water and reaching sizes of 350nm. Several studies reported that agglomeration is related to lower toxicity, as demonstrated by Laban et al. (2010), where fish embryos of *Pimephales promelas* were exposed to stirred and sonicated AgNPs and reported a higher toxicity for sonicated AgNPs, possibly due to the presence of lower size particles/aggregates/agglomerates. Nanoparticles aggregates may have lower toxicity as they are unable to penetrate into cells and be internalized by organisms (Snell and Hicks, 2009 and Ribeiro et al., 2014), or due to their decrease in area (compared to the individualized initial particles) and the release of ions is also lower.

The agglomeration of NPs will favor the removal of NPs from the water column and their deposition into the sediments (Navarro et al., 2008 and Hedberg et al., 2014). Up to 90% of all AgNPs can be removed within 48 hours from the water column (Griffitt et al., 2008), but this timeframe varies between different NPs, and the test system present, as demonstrated by Li and Lenhart (2012), where similar values of dissolved silver were present after 6 hours and 15 days.

In the present study, chironomid larvae exposed to AgNPs seem to be able to reach the fourth instar. Posteriorly failure of emergence occured and delay of emergence day. Similar results were related to mortality occurred in this stage and in the transition to the pupal stage, with several studies reporting perceptible delays for emergence of imagoes exposed to several like aromatic compounds and particularly metals such as mercury in water (Bleeker et al., 1999; Pascoe et al., 1989; Vogt et al., 2007a; Azevedo-Pereira et al., 2010). The weight of chironomids was unresponsive however there was sexual differentiation. This discrepancy was to be expected, as males are of smaller size.

The delay of development at 28 days and no early reports of effects in growth rates can be explained by two factors: increasing toxicity of AgNPs due to ionic release and/or vulnerability of chironomids during metamorphosis. The higher toxicity of AgNPs at the end of the experiment can also be related to an oxidation related phenomena, as AgNPs are prone to oxidation in aerated media increasing their toxicity from their initial state (Lok et al., 2007), and was reported to occur marginally with the AgNPs used (Ribeiro et al., 2014). On the other hand, pupal exuvie are reported to concentrate more metals than the emerged adults. This indicates that during metamorphosis metals accumulate in the pupal skin, which may be a mean to eliminate excess metals before emergence (Kosalwat and Knight, 1987 and Lagrana et al., 2011). The process of accummulation is energy consuming and may take longer, delaying emergence when exposed to AgNPs. Failure to eliminate excess metals can lead to the failure of pupation. These effects may likely happen through the modification of expression profiles of the ecdysone receptor genes. This genes control hormone regulation and are prone to altered modulation when in presence of environmental chemicals. This has been reported in other species such as the nematode worm *Caenorhabditis elegans* it affects longevity (Gáliková et al., 2011); in *D. melanogaster*

it is known to play essential roles in triggering and regulating metamorphosis (Liu et al., 2014); and additionally in the corn stalk borer, *Sesamia monogrioides* (Lepidoptera: Noctuidae) there were reported disturbances in development and metamorphosis (Kontogiannatos et al., 2014). In *C. riparius* AgNPs and other compounds were already reported to affect ecdysteroid-regulated pathways leading to reduction in the rates of total adult emergence and emergence failures (Nair and Choi, 2012). Such mechanism could also hindered organisms in the present study.

Regarding $AgNO_3$, the highest concentration used was 90 μg $Ag.L^{-1}$, and no sublethal effects were observed throughout the 28 days, being therefore difficult to compare the toxicity at this life stage for both Ag forms. From preliminary trials, if concentrations were increased high mortality rates would be observed bringing no adequate sublethal data.

2.6 Conclusion

C. riparius egg masses showed high resistance to silver exposure. The eggs endurance may be potentiated by their gelatinous matrix and the egg cell exterior. The low values for toxicity in the acute test were to be expected as it is stated that the first stages of the life cycle of benthic organisms are less resistant to AgNPs (Gauss et al., 1985 and García-Alonso et al., 2014). Emergence and time to emerge could have been affected mainly through ecdysteroid-regulated pathways, nevertheless further studies are needed to verify this hypothesis. Results indicate that metamorphosis and the first instar larvae are the most vulnerable stages in the chironomids life cycle, when in a contaminated water environment.

Further studies are needed to assert, what induces toxicity, potential effects in hormone pathways, probable intergenerational effects, and a reliable comparison with other silver species such as free silver ions.

2.7 References

ASGHARI S., JOHARI S.A., LEE J.H., KIM Y.S., JEON Y.B., CHOI H.J., MOON M.C. and YU I.J., 2012. Toxicity of various silver nanoparticles compared to silver ions in Daphnia magna. Journal of Nanobiotechnology, 10, 14.

ASTM, 2010. Standard Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates. Report E 1706 – 05. American Standards for Testing and Materials, Philadelphia, P.A.

AZEVEDO-PEREIRA H.M.V.S., LEMOS M.F.L. and SOARES A.M.V.M., 2010. Effects of Mercury on Growth, Emergence and Behavior of *Chironomus riparius* Meigen (Diptera: Chironomidae). Archives of Environmental Contamination and Toxicology, 59, 216-224.

AZEVEDO-PEREIRA H.M.V.S., LEMOS M.F.L. and SOARES A.M.V.M., 2011. Behavior and Growth of *Chironomus riparius* Meigen (Diptera: Chironomidae) under Imidacloprid Pulse and Constant Exposure Scenarios. Water, Air, & Soil Pollution, 219, 215-224.

BAI W., ZHANG Z., TIAN W., HE X., MA Y., ZHAO Y. and CHAI Z., 2009. Toxicity of zinc oxide nanoparticles to zebrafish embryo: a physicochemical study of toxicity mechanism. Journal of Nanoparticle Research, 12, 1645-1654.

BENN T.M. and WESTERHOFF P., 2008. Nanoparticle silver released into water from commercially available sock fabrics. Environmental Science & Technology 42:4133-4139.

BELL R.A. and KRAMER J.R., 1999. Structural chemistry and geochemistry of silver-sulfur compounds: critical review. Environmental Toxicology and Chemistry, 18:9–22.

BIANCHINI A. & WOOD C.M., 2003. Mechanism of acute silver toxicity in Daphnia magna. Environmental Toxicology and Chemistry, 22, 1361–1367.

BLEEKER E.A.J., LESLIE H.A., GROENENDIJK D., PLANS M. and ADMIRAAL W., 1999. Environmental Toxicology and Chemistry, 18(8), 1829–1834.

BROWN D.M., DONALDSON K., BORM P.J., SCHINS R.P., DEHNHART M., GILMOUR P., JIMENEZ L.A. and STONE V., 2004. Calcium and ROS-mediated activation of transcription

factors and TNF-alpha cytokine gene expression in macrophages exposed to ultrafine particles. American Journal of Physiology Lung Cellular and. Molecular Physiology, 286(2), L344-53.

BURKHARDT M., ENGLERT A., ITEN R. and SCHÄRER S. 2011. Entsorgung nanosilberhaltiger Abfälle in der Textilindustrie - Massenflüsse und Behandlungsverfahren University for Applied Sciences, Rapperswil. (Acessed 3 of March, 2015) http://www.umtec.ch/uploads/tx_hsrpm/120310_Studie_NanAgTex_Abschlussbericht_BAFU_Final_01.pdf.

CALL D.J., POLKINGHORNE C.N., MARKEE T.P., BROOKE LT, GEIGER DL, GORSUCH JW and ROBILLARD K.A., 1999. Silver Toxicity to *Chironomus Tentans* in Two Freshwater Sediments. Environmental Toxicology and Chemistry, 18(1), 30–39.

DE SCHAMPHELAERE K.A.C., VASCONCELOS F.M., TACK F.M.G., ALLEN H.E. and JANSSEN C.R., 2004. Effect of dissolved organic matter source on acute copper toxicity to Daphnia magna. Environmental Toxicology and Chemistry, 23, 1248–1255.

DOBIAS J. and BERNIER-LATMANI R., 2013. Silver Release from Silver Nanoparticles in Natural Waters. Environmental Science and Technology, 47, 4140−4146.

EDMOND C., 2014. Toxicokinetics and interaction of nanoparticles within biological matrices in Nanotechnology and human health. Boca Raton: CRC Press, 2014 ISBN 13:978-0-8493-8144-7. Chap. 9, p. 165-177.

FARIA M.S., RE A., MALCATO J., SILVA P.C., PESTANA J., AGRA A.R., NOGUEIRA A.J. and SOARES A.M.V.M., 2006. Biological and functional responses of in situ bioassays with Chironomus riparius larvae to assess river water quality and contamination. Science of the Total Environment, 371 (1-3), 125-137.

FROUZ J., ALI A., and LOBINSKE R.J., 2002. Influence of temperature on developmental rate, wing length, and larval head capsule size of pestiferous midge *Chironomus crassicaudatus* (Diptera: Chironomidae). Journal of Economic Entomology, 95, 699–705.

GÁLIKOVÁ M., KLEPSATEL P., SENTI G. and FLATT T., 2011. Steroid hormone regulation of C. elegans and Drosophila aging and life history. Experimental Gerontology, 46, 141 – 147.

GARCÍA-ALONSO J., RODRIGUEZ-SANCHEZ N., MISRA S.K., VALSAMI-JONES E., CROTEAU M., LUOMA S.N. and RAINBOW P.S., 2014. Toxicity and accumulation of silver nanoparticles during development of the marine polychaete *Platynereis dumerilii*. Science of the Total Environment, 476–477, 688–695.

GAUSS J.D., WOODS P.E., WINNER R.W. and SKILLINGS J.H., 1985. Acute toxicity of copper to three life stages of Chironomus tentans as affected by water hardness-alkalinity. Environmental Pollution – Series A: Ecological and Biological, 37, 149–157.

GILLIS P.L. and WOOD C.M., 2008. The effect of extreme waterborne cadmium exposure on the internal concentrations of cadmium, calcium, and sodium in Chironomus riparius larvae. Ecotoxicology and Environmental Safety, 71, 56–64.

GONG Z.J.P., XIE P. and LI Y.L., 2002. Effect of temperature and photoperiod on hatching of eggs of *Tokunagayusurika akamusi* (Tokunaga) (Diptera: Chironomidae). Journal of Freshwater Ecology, 17, 169–170.

GORTH D.J., RAND D.M. and WEBSTER T.J., 2011. Silver nanoparticle toxicity in Drosophila: size does matter. International Journal of Nanomedicine, 6, 343–350.

GRIFFITT R.J., LUO J., GAO J., BONZONGO J. and BARBER D.S., 2008. Nanomaterials in the Environment: Effects of Particle Composition and Species on Toxicity of Metallic Nanomaterials in Aquatic Organisms. Environmental Toxicology and Chemistry, 27(9), 1972–1978.

HEDBERG J., SKOGLUND S., KARLSSON M., WOLD S., WALLINDER I.O. & HEDBERG Y., 2014. Sequential Studies of Silver Released from Silver Nanoparticles in Aqueous Media Simulating Sweat, Laundry Detergent Solutions and Surface Water. Environmental Science and Technology, 48, 7314−7322

HEIQ Materials Ltd. Personal communication. Switzerland: Zurich; 2006.

HORWELL C.J. and Baxter P.J., 2006. The respiratory health hazards of volcanic ash: a review for volcanic risk mitigation. Bulletin Volcanology, 69, 1-24.

KIM B., PARK C., MURAYAMA M. and HOCHELLA M.F.JR., 2010. Discovery and Characterization of Silver Sulfide Nanoparticles in Final Sewage Sludge Products. Environmental Science and Technology, 44, 7509–7514.

KLOEPFER J.A., MIELKE R.E. and NADEAU J.L., 2005. Uptake of CdSe and CdSe/ZnS quantum dots into bacteria via purine-dependent mechanisms. Applied and Environmental Microbiology, 71, 2548–2557.

KUMAR R., HOWDLE S. and MÜNSTEDT H., 2005. Polyamide/silver antimicrobials: effect of filler types on the silver ion release. Journal of Biomedical Materials Research Part B: Applied Biomater, 75B, 311–9.

KONTOGIANNATOS D., SWEVERS L., ZAKASIS G. and KOURTI A., 2015. The molecular and physiological impact of bisphenol A in *Sesamia nonagrioides* (Lepidoptera: Noctuidae). Ecotoxicology, 24(2), 356-367.

KOSALWAT P. and KNIGHT A.W., 1987. Chronic Toxicity of Copper to a Partial Life Cycle of the Midge, Chironomus decorus. Archives of Environmental Contamination and Toxicology, 16, 283-290.

LABAN G., NIES L.F., TURCO R.F., BICKHAM J.W. and SEPÚLVEDA M.S., 2010. The effects of silver nanoparticles on fathead minnow (*Pimephales promelas*) embryos. Ecotoxicology, 19, 185–195.

LAGRANA C., APODACA D. and DAVID C.P., 2010. Response of Chironomids to Cu^{2+}, Cd^{2+} and Pb^{2+} Metal Ions Using the 96-hour Toxicity Test and Evaluating Different Factors that Affect Metal Accumulation. Journal of Trends in Chemistry, 1(1), 8-13.

LAGRANA C.C., APODACA D.C. and PRIMO C.D., 2011. Chironomids as Biological Indicators of Metal Contamination in Aquatic Environment. International Journal of Environmental Science and Development, 2(4), 306-310.

LARRAIN A., RIVEROS A., BAY-SCHMITH E. and ROA R., 1997. Evaluation of three larval instars of the midge *Chironomus petiolatus* as bioassay tools using a computationally intensive statistical algorithm. Archives of Environmental Contamination and Toxicology, 33, 407–414.

LEAL H. , SOETER A.M., KOOLS S.A.E., KRAAK M.H.S., PARSONS J.R., TEMMINK H., ZEEMAN G. and BUISMAN C.J.N., 2012. Ecotoxicological assessment of grey water treatment systems with Daphnia magna and *Chironomus riparius*. Water Research, 46, 1038-1044.

LI X. and LENHART J.J., 2012. Aggregation and Dissolution of Silver Nanoparticles in Natural Surface Water. Environmental Science & Technology, 46, 5378–5386.

LIU J. and HURT R.H., 2010. Ion release kinetics and particle persistence in aqueous nano-silver colloids. Environmental Science & Technology, 44, 2169–75.

Liu T., Li Y., Zhao X., Zhang M. and Gu W., 2014. Ethylparaben affects lifespan, fecundity, and the expression levels of ERR, EcR and YPR in Drosophila melanogaster. Journal of Insect Physiology, 71, 1–7.

LOK C.N., HO C.M., CHEN R., HE Q.Y., YU W.Y., SUN H., TAM P.K.H., CHIU J.F. and CHE C.M., 2007. Silver nanoparticles: partial oxidation and antibacterial activities. Journal of Biological Inorganic Chemistry, 12, 527–534.

LORENZ C., L. WINDLER L., VON GOETZ N., LEHMANN R.P., SCHUPPLER M., HUNGERBUHLER K., HEUBERGER M. and NOWACK B., 2012. Characterization of silver release from commercially available functional (nano) textiles. Chemosphere, 89, 817–824.

LUOMA S.N., 2008. Silver Nanotechnologies and the Environment: Old Problems or New Challenges? Project on Emerging Nanotechnologies. Woodrow Wilson Centre, Washington, DC, USA.

MARINKOVIĆ M., DE BRUIJN K., ASSELMAN M., BOGAERT M., JONKER M.J., KRAAK M.H.S. and ADMIRAAL W., 2012. Response of the nonbiting midge *Chironomus riparius* to

multigeneration toxicant exposure. Environmental Science & Technology, 46, 12105-12111.

MISRA S.K., DYBOWSKA S., BERHANU D., LUOMA S.N. and VALSAMI-JONES E., 2012. The complexity of nanoparticle dissolution and its importance in nanotoxicological studies. Science of the Total Environment, 438, 225–232.

MORONES J.R., Elechiguerra,J.L., Camacho A., Holt K., Kouri J.B., Ramirez J.T. and Yacaman M.J., 2005. The bactericidal effect of silver nanoparticles. Nanotechnology, 16, 2346–2353.

NANOSILVER SUSPENSION AX 06HX, AMEPOX http://www.amepox-mc.com/cache/files/1298757566_NanoSilver_Suspension_AX_06Hx.pdf Accessed March 2015

NAIR P.M.G. and CHOI J., 2012. Modulation in the mRNA expression of ecdysone receptor gene in aquatic midge, Chironomus riparius upon exposure to nonylphenol and silver nanoparticles. Environmental Toxicology and Pharmacology. 33, 98 – 106.

NAVARRO E., BAUN A, BEHRA R., HARTMANN N.B., FILSER J., MIAO A.J., QUIGG A., SANTSCHI P.H. and SIGG L., 2008. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. Ecotoxicology, 17,372-386.

NEBEKER A.V., CAIRNS M.A. and WISE C.M., 1984. Relative sensitivity of Chironomus tentans life stages to copper. Environmental Toxicology and Chemistry, 3, 151–158.

NOWAK C., VOGT C., BARATEIRO J.D.I. and SCHWENK K., 2007. Genetic Impoverishment in Laboratory Cultures of the test Organism *Chironomus riparius*. Environmental Toxicology and Chemistry, 26(5), 1018–1022.

OBERDÖRSTER G., OBERDÖRSTER E. and OBERDÖRSTER J., 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environmental Health Perspectives, 113, 823–839.

OECD, 2004. Test guideline 219. Sediment-water chironomid toxicity test using spiked water. OECD Guidelines for the testing of chemicals.

OECD, 2010. Test guideline 233. Sediment-water chironomid life-cycle toxicity test using spiked water or spiked sediment. OECD Guidelines for the testing of chemicals.

OECD, 2011. Test guideline 235. Chironomus sp., acute immobilisation test. OECD Guidelines for the testing of chemicals.

PAL S., TAK Y.K. and SONG J.M., 2007. Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium Escherichia coli. Applied and Environmental Microbiology, 73, 1712–1720.

PARK E.J., YI J., KIM Y., CHOI K. and PARK, K., 2010. Silver nanoparticles induce cytotoxicity by a Trojan-horse type mechanism. Toxicology In Vitro, 24, 872–878.

PASCOE D., WILLIAMS K.A. and GREEN D.W.J., 1989. Chronic toxicity of cadmium to *Chironomus riparius* Meigen—effects upon larval development and adult emergence. Hydrobiologia, 175, 109 – 115.

PESTANA J.L.T., ALEXANDER A.C., CULP J.M., BAIRD D.J., CESSNA A.J. and SOARES A.M.V.M., 2009. Structural and functional responses of benthic invertebrates to imidacloprid in outdoor stream mesocosms. Environmental Pollution, 157(8–9), 2328-2334.

PETERS A., VERONESI B., CALDERÓN-GARCIDUEÑAS L., GEHR P., CHEN L.C., GEISER M., REED W., ROTHEN-RUTISHAUSER B., SCHÜRCH S. and SCHULZ H., 2006. Translocation and potential neurological effects of fine and ultrafine particles a critical update. Particle and Fibre Toxicology, 3, 13.

PHILBROOK N.A., WINN L.M., AFROOZ A.R.M.N., SALEH N.B. and WALKER V.K., 2011. The effect of TiO2 and Ag nanoparticles on reproduction and development of Drosophila melanogaster and CD-1 mice. Toxicology and Applied Pharmacology, 257(3), 429–436.

POSTMA J.F., KYED M. and ADMIRAAL W., 1995. Site specific differentiation in metal tolerance in the midge Chironomus riparius (Diptera, Chironomidae) Hydrobiologia, 315, 159-165.

RIBEIRO F., GALLEGO-URREA J.A., JURKSCHAT K., CROSSLEY A., HASSELLOV M., TAYLOR C., SOARES A.M.V.M and LOUREIRO S., 2014. Silver nanoparticles and silver nitrate induce high toxicity to *Pseudokirchneriella subcapitata*, *Daphnia magna* and *Danio rerio*. Science of the Total Environment, 466-467, 232-241.

RIBEIRO F., GALLEGO-URREA J.A., GOODHEAD R.M., VAN GESTEL C.A., MOGER J., SOARES A.M.V.M. and LOUREIRO S., 2014. Uptake and elimination kinetics of silver nanoparticles and silver nitrate by *Raphidocelis subcapitata*: The influence of silver behaviour in solution. Nanotoxicology, 1-10.

ROUSCH J.M., SIMMONS T.W., KERANS B.L. and SMITH B.P., 1997. Relative acute effects of low pH and high iron on the hatching and survival of the water mite (*Arrenurus manubriator*) and the aquatic insect (*Chironomus riparius*). Environmental Toxicology and Chemistry, 16 (10), 2144-2150.

SCOW K., GOYE M., NELKEN L., PAYNE E., SATERSON K., WALKER P. and WOOD M. 1981. An exposure and risk assessment for silver. Technical Report. US Environmental Protection Agency, Washington, DC.

SNELL T.W. and HICKS D.G., (2009). Assessing Toxicity of Nanoparticles Using *Brachionus manjavacas* (Rotifera). Environmental Toxicology, 26(2), 146-152

SPSS (2008). SigmaPlot for Windows (version 11.0). Science California, USA.

VOGT C., PUPP A., NOWAK C., JAGODZINSKI L.S., BAUMMANN J., JOST D., OETKEN M. and OEHLMANN J., 2007. Interaction between genetic diversity and temperature stress on life-cycle parameters and genetic variability in midge *Chironomus riparius* populations. Climate Research, 33, 207-214.

VOGT C., BELZ D., GALLUBA S., NOWAK C., OETKEN M. and OEHLMANN J., 2007A. Effects of cadmium and tributyltin on development and reproduction of the non-biting midge *Chironomus riparius* (Diptera) - baseline experiments for futuremulti-generation studies. Journal of Environmental Science and Health Part A, 42(1), 1–9.

WAISSI-LEINONEN, G. C., PETERSEN E.J., PAKARINEN K., AKKANEN J., LEPPANEN M.T. and KUKKONEN J.V., 2012. Toxicity of fullerene (C60) to sediment-dwelling invertebrate Chironomus riparius larvae. Environmental Toxicology and Chemistry, 31(9), 2108-2116.

WILLIAMS K.A., GREEN D.W.J., PASCOE C. and GOWER D.E., 1986.The acute toxicity of cadmium to different larval stages of *Chironomus riparius* (Diptera: Chironomidae) and its ecological significance for pollution regulation. Oecologia, 70, 362–366.

XIA T., KOVOCHICH M., BRANT J., HOTZE M., SEMPF J., OBERLEY T., SIOUTAS C., JI Y., WIESNER M.R. and NEL A.E., 2006. Comparison of the Abilities of Ambient and Manufactured Nanoparticles to Induce Cellular Toxicity According to an Oxidative Stress Paradigm. Nano Letters, 6(8), 1794 -1807.

Chapter 3

Silver nanoparticles' toxicity to *Chironomus riparius* in contaminated sediments

3. Silver nanoparticles toxicity to *Chironomus riparius* in contaminated sediments

3.1 Abstract

Keywords: Silver nanoparticles (AgNPs), *Chironomus riparius*, sediment, toxicity

Sediments act as the ultimate depository basin of different chemical compounds and silver nanoparticles (AgNPs) are no exception. To evaluate the toxicity of silver compounds throughout the life-cycle of chironomids spiked sediment was used as exposure route and survival, larval growth, emergence, weight, fertility and sex ratio were assessed along with four biomarkers associated with important physiological functions (catalase – CAT; acetylcholinesterase − Ache; glutathione-S-transferase − GST and lipid peroxidation − LPO). In this study a decrease on the growth rate of both head and body length was observed after ten days of chronic exposure to AgNPs spiked sediment, increasing with concentrations. The EC_{50} values regarding head and body growth rate were both extrapolated (>200 $mg.Kg^{-1}$). Survival at 10 days was also affected but again no LC_{50} could be depicted. The longer exposure of 28 days to AgNPs induced an emergence decrease, with an EC_{50} of 122.19 $mg.Kg^{-1}$, whilst time to emerge and weight were unresponsive. An increase in activity was only observable in CAT and GST after 24h. The endpoints of the parental generation were compatible with the values obtained during the 28 day toxicity test. The number of egg masses laid per female showed a dose response relationship (EC_{50} 13.10 $mg.Kg^{-1}$) along with their fertility (EC_{50} of 70.21 $mg.Kg^{-1}$) likewise presented the same pattern. In the filial generation an impairment was noted in emergence of adults (EC_{50} of 49.20 $mg.Kg^{-1}$) and the time to emergence diminished (EC_{50} 24.50 $mg.Kg^{-1}$). The toxicity of silver maybe lower due to binding of complexes in the sediment.

3.2 Introduction

The total use and consumption of nanomaterials worldwide reached 225,060 metric tons in 2014 and is estimated to increase at a rate of 21.1% for the period between 2014 and 2019, reaching nearly 584,984 metric tons in 2019 (BCC Research, 2014). These values will surely lead to enlarged release of nanomaterials to the environment, mainly into the aquatic medium, occurring interchange between the water column, sediments and the biota (Van Cappellen and Wang, 1995; Nowack and Bucheli, 2007; Dobias and Bernier-Latmani, 2013).

The first attempts to assess toxicity in sediments were undergone at the end of 1970s, as a by-product of dredging operations (Burton, 1991), along with the need to evaluate the extent of contamination present in sediments (Rosiu et al., 1989). For several years bioassessments of toxicity in sediments have been regulated (EPA, 1991; EPA 1993) and are essential in ecotoxicology. Concentration of metals in benthic organisms is directly correlated with concentration of metals present in sediments and in the water column (David, 2003). However, there are species that show a higher adaptation to contamination, and are able to maintain viable reproducing populations in environments extremely deteriorated, like Chironomids (Gabriels et al., 2010). A debate is still ongoing on how exactly chironomids are able to withstand long term toxicity. The debate revolves around if toxicant resistance is due to phenotypic plasticity (Postma and Davids, 1995 and Marinkovic et al., 2012), genetic adaptation (Morgan et al., 2007 and Vedamanikam and Shazilli, 2008) or both models are at play (Miller and Hendricks, 1996). Chironomids are good model organisms to assess sediment toxicity as they are continuously exposed to contaminants present in sediment from the 1st instar after 48 hours and until pupation. In addition, chironomids may retain nanoparticulate matter in the gut through their processes of burrowing and while searching for food (Postma et al., 1996 and Snell and Hicks, 2009). The effects of silver sediment toxicity in chironomids can thus be weighed by assessing growth patterns, a well-documented sensitive endpoint. Growth patterns will be affected by any disruption at the biochemical, physiological or behavioral level; when such

disruptions occur they can reverberate in higher trophic levels unbalancing the ecosystem (Dyar, 1890; Wentsel et al., 1977; Rosiu et al., 1989). It has been predicted that environmental stressors that induce deregulations in growth, generating smaller sized Chironomids, can lead to reduced and failure reproduction (Rodrigues et al., 2015). Additionally adults reared in contaminated environments can potentially transmit (epi)genetic modified patterns to the next generation (Kosalwat and Knight, 1987). As silver nanoparticles are reported to act through the disruption of cell membranes, which can potentiate free silver ions and nanoparticles deliver into the cell (Luoma, 2008 and Park et al., 2010), this will enhance the levels of reactive oxygen species (ROS) (Klaine et al., 2008) or induce oxidative stress when present in high quantities. Oxidative stress is characterized by damaging proteins, affecting lipid peroxidation, inducing DNA disruption and the occurrence of oxidative stress can be assessed by the use of biomarkers of effect (Walters et al., 2014).

The present study intends to assess the effects of sediment contamination with AgNPs to the midge *C. riparius* throughout their life-cycle. Different exposures were devised to achieve the objectives: from chronic exposure of first instar larvae, life-cycle test and biomarker assessment trials. To discriminate between different species of silver, trials were run using both AgNPs and $AgNO_3$ exposures.

3.3 Material and Methods

3.3.1 Test Organism

Chironomus riparius individuals used in all bioassays were obtained from a well-established culture maintained at the Department of Biology, University of Aveiro. This culture is kept in a transparent acrylic box (120cm × 60cm × 40cm), which allows enough space for swarming and copulation of adults, as well as boxes with sand and artificial culture media for larvae and pupa, enabling the whole life cycle to be maintained (OECD,

2004). The aquaria for the larval and pupa stages are constantly aerated and hold a 2 cm layer of burned commercial sand (<1 mm), and approximately 2.5 L of reconstituted ASTM hard water. The culture is maintained at 20±2 °C, 16: 8 h light: dark photoperiod, with an intensity of approximately 1000 lux. Water and sediment are renewed every week and every fortnight respectively, and the larvae are fed every other day with a suspension of TetraMin® (Tetrawerke, Germany), in a weight ratio of 20mL: 1g (OECD, 2011). These conditions were also applied to every test performed in the present work.

3.3.2 Test Substances

In the present study it was used silver nitrate ($AgNO_3$), supplied by Sigma-Aldrich (St. Louis, MO), with 99% purity (CAS 7761-88-8) and an ultrapure water dispersion of AgNPs, with an initial concentration of 1000 ppm, supplied by AMEPOX Enterprise). Silver nanoparticles in suspension had an initial size between 3 and 8 nm, with an artificial alkane protection layer and a zeta potential of 33mV (NanoSilver Suspension AX 06Hx, 2015). Both reagents were kept in darkness and room temperature during storage. The beakers used in all procedures were of chemical inert glass, and were cleansed in an acid wash, as standard practice (Handy et al., 2012). During the experiments artificial sediment was used to avoid potential contaminations and for a better comparison with other studies due to its standardization and internationally availability (Ribeiro et al., 1999). The artificial sediment used was adjusted to a pH of 6.25, with $CaCO_3$, and consisted for 75% of quartz sand, 20% of kaolin with a 0.1-0.4 µn particle size (Sigma Co.) and 5% of α-cellulose (Sigma Co.) (OECD, 2004). The sediment was spiked with an appropriate volume of a stock solution/dispersion to the final desired concentration. The stock solution/dispersion was prepared previously with deionized water. The sediments were then stirred to ensure homogeneity. Afterwards, and in order to assure that after water addition there was no movement of Ag from the sediment to the water column, a cotton disk was deposited on the top sediment while ASTM artificial water was poured, and immediately removed at the

end of the procedure. The system was allowed to rest 48h before the insertion of larvae and the aeration was only provided 24 hours after the first instar larvae were added.

3.3.3 Characterization of test compounds

An attempt to characterize AgNPs and $AgNO_3$ in the test media was undergone by Transmission Electron Microscopy (TEM) imaging. The highest concentrations used for AgNPs (200 $mg.Kg^{-1}$) and $AgNO_3$ (25 $mg.Kg^{-1}$), were diluted to 0.1 $mg.mL^{-1}$ in the test media. Afterwards a droplet was positioned on a holey carbon coated Cu TEM grid and allowed to dry at room temperature. Experiments were carried out on a JEOL 2010 analytical TEM (resolution 0.19 nm, electron probe size down to 0.5 nm and maximum specimen tilt ±10° along both axes). The instrument is equipped with an Oxford Instruments LZ5 windowless energy dispersive X-ray spectrometer (EDS) controlled by INCA.

3.3.4 Chronic test

A chronic test was carried out using two different assessment times of exposure: 10 day and 28 days. Fifty grams of sediment were distributed, per replicate, into 200mL flasks. Sediment was spiked in situ and allowed to dry at room temperature (according to the OECD guidelines). As described above, later 150mL of artificial water ASTM was added per replicate. The concentrations used for $AgNO_3$ were chosen based on previous results obtained in preliminary tests (data not shown) and were of 1.57, 3.13, 6.25, 12.5, 25 µg per gram of formulated sediment The maximum concentration used for the exposure with silver nanoparticles was the highest possible considering the concentration of the suspension provided by Amepox. The concentrations used were 12.5, 25, 50, 100 and 200 $µg.g^{-1}$ of AgNPs. For each treatment a negative control and 7 replicates with nine larvae were used (OECD, 2004). Endpoints were assessed at two different times: 10 and 28 days.

To assess endpoints at 10 days an additional number of replicates was sacrificed, chironomids were exposed to Ag via sediment, and at the end of 10 days survival and growth rates were measured. (De Haas et al. 2006). The growth rate was calculated using both head width and body size. The measurements of head capsule were adapted from previous studies to determine variations in reaching the development instar stages (Watts and Pascoe, 2000). To achieve this, an initial group of larvae was sacrificed to ensure a comparison of the final size to its initial non-exposed mean. All measurements were made in a stereo microscope (MS5, Leica Microsystems, Houston, USA) fitted with a calibrated eye-piece micrometer. To estimate the growth rates over 10 days it was used this equation adapted from (Leal et al., 2012):

$$\text{Growth rates} = \frac{(\text{mean final size}) - (\text{mean initial size})}{10 \text{ days}}$$

Development time, percentage of emerged chironomids and dry weight of adults were used as endpoints. Once larvae reached adulthood their gender was identified and kept in alcohol (70%). Afterwards midges were allowed to dry at room temperature and were weighed in a microbalance.

3.3.5 Life cycle test

A contaminated artificial sediment – clean water system was prepared two days prior to the experiment following the already described procedures in the chronic tests. First instar larvae from the parental generation (F0) were assorted randomly into 200 mL glass beakers, 9 individuals in each vessel and 24 replicates per concentration, including a negative control. As adults emerged they were assorted to breeding cages for the respective concentration and replicate, where they were able to swarm, mate and lay eggs. The egg masses were transferred into 50 ml beakers and allowed to hatch (Streloke and

Köpp, 1995; and Vogt et al., 2007). From the hatched egg ropes it was obtained the filial generation (F1) that was also exposed through similar procedures, as F1 larvae were transferred into newly prepared vessels with spiked sediment for an additional 28 day exposure.

Regarding the toxicity of AgNPs spiked sediments across generations the emergence success, time to emerge and sex ratio in F0 and F1 were reported. Additionally, it was assessed the fertility of egg masses only laid by the F0 generation. The fertility of egg ropes was evaluated through the course of six days, and they were fertile when at least one third of its eggs hatched. The total number of females added to the breeding cage was used to calculate the number of egg ropes per female and the sex ratio of the chironomid population (OECD, 2010).

3.3.6 Biomarkers test

In order to understand some general mechanistic effects of Ag and its different forms under Ag spiked sediment exposures, tests were performed with a 24 hour exposure of 4^{th} instar larvae. No food was provided during the trial. For the exposures 500ml crystallizing dishes containing ASTM hard water and Ag spiked artificial sediment were used, with concentrations of 50, 100 and 200 $mg.Kg^{-1}$ and a negative control. For each treatment, 7 replicates with 15 organisms each were used.

After exposure, larvae were collected and quickly dried on filter paper, weighed, frozen in liquid nitrogen and kept at – 80 ºC. Samples were homogenized by sonication in ice in equal proportions by adding 0.2 M K-Phosphate buffer, pH 7.4 1600 µl; the homogenate was afterwards centrifuged for 20 min at 10,000 g (4 ºC). Then, four enzymes were chosen and measured in order to assess potential induced oxidative stress glutathione s-transferase (GST), catalase (CAT), acetylcholinesterase (Ache), and lipid peroxidation (LPO). For the determination of LPO 200 µL tissue homogenate was separated into a microtube with 4 µL of butylated hydroxytoluene (BHT) and afterward 1000µL of TBA

0.73% solution. The samples were placed in an oven at 100ºC for 1 hour. Posteriorly they were centrifuged at 11500 rpm for 5 minutes at 25ºC. The supernatant was transferred to a microplate and the absorbance read at 535 nm (Bird and Draper, 1984). CAT activity was determined by adding PMS to the supernatant and measuring decomposition of the substrate H_2O_2 at 240 nm during 1 minute (Clairborne, 1985). GST activity was determined by adding a glutathione solution 10 mM with 1-chloro-2,4-dinitrobenzene to the samples. Absorbance was read at 340 nm, every 20 seconds for 5 minutes (Habig et al., 1974). To determine AChE acetiltiocoline and DTNB were added to the supernatant, and the reaction was monitored at 412nm (Ellman et al. (1961) adapted to microplate by Guilhermino et al. (1996)).

3.3.7 Statistical Analysis

To determinate variations amongst the treatments performed and controls a One Way ANOVA Analysis of Variance was used. Whenever raw data did not passed the normality test and transformations were not able to correct for normality, a Kruskal-Wallis One Way Analysis of Variance on Ranks was carried out. When significant differences were present the Dunn's Method or the Holm-Sidak's Method were carried out to discriminate statistical differences between treatments (SPSS 2008). The 50% effective concentration (EC_{50}) values were calculated using a nonlinear regression with a sigmoidal function, using always the one showing the best adjustment.

3.4 Results

Testing for physical characters was necessary to validate the experiments. The values obtained for pH, conductivity and dissolved oxygen remained stable and mostly unaltered throughout the assays and within the parameters established by the chironomid toxicity test guideline. The pH was kept between 6 and 9 and water temperature did not differ by more than 1 ºC throughout the experiment (OECD, 2004).

3.4.1 Characterization of test compounds

The analysis performed by TEM measurements revealed the presence of AgNPs aggregates, with approximately 50–150 nm in diameter (Fig. 3.1.); and a probable presence of silver sulphide compounds (Fig. 3.2.). Additionally, signals of aluminum and silica were found, most likely related to sediment particles. TEM imaging of the $AgNO_3$ sample did not show any presence of silver, probably due to the low concentrations used.

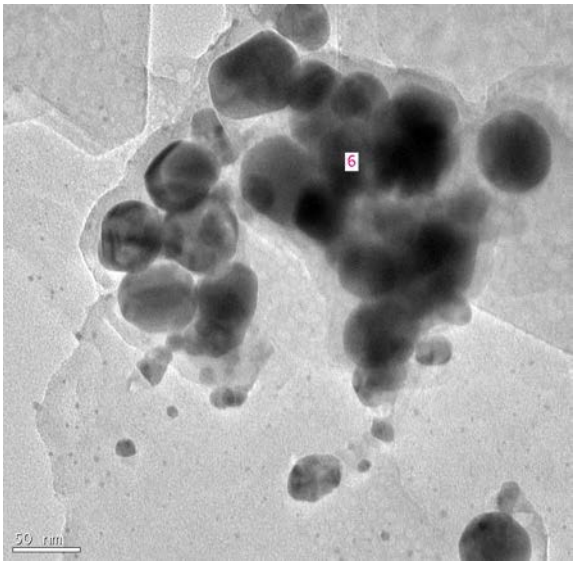

Fig. 3.1. TEM image of silver nanoparticles. Area 6 shows nanoparticles agglomerates and the organic film on the outside.

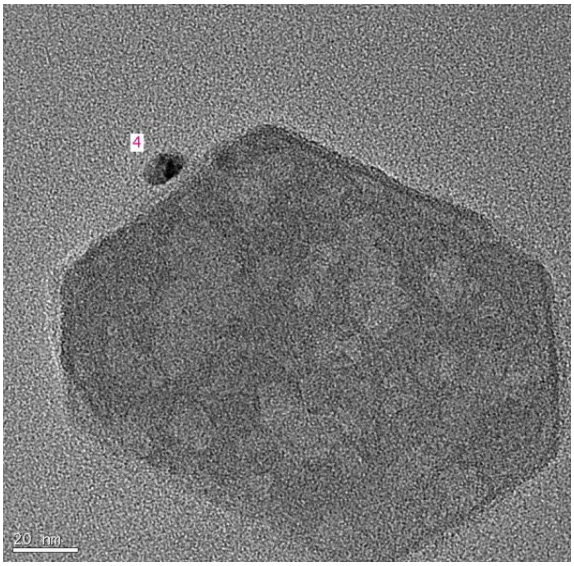

Fig. 3.2. TEM image of silver nanoparticles. Area 4 shows probable silver sulphide particles.

3.4.2 Chronic tests

Growth rate test

The presence of AgNPs in the sediment induced a decrease on chironomids' survival at 10 days (Fig. 3.3.) in the highest concentration used, with an average of 30% more mortality than the control. The NOEC was 100 mg.Kg^{-1}, and LOEC of 200 mg.Kg^{-1} (Kruskal–Wallis ANOVA on Ranks, H=14.84, df=5, $p<0.05$) and the LC_{50} was derived as higher than the highest concentration used (extrapolated to 229.84 ± 24.17 mg.Kg^{-1}). The body growth rate was significantly affected showing a clear dose response pattern (Fig. 3.4.); whilst the mean larval growth rate in controls was close to 1.1mm.day-1, at the highest concentrations of AgNPs it was only 0.6 mm.day-1 (One way ANOVA, F5, 36=40.95; $p<0.05$; Holm-Sidak's test, $p<0.05$). The LOEC coincided with the lowest dose tested of 12.5 mg.Kg-1, and a mean body growth rate reduction of 50% was expected to occur at concentrations of AgNPs higher than the ones used in the present study (extrapolated to 334.03 ± 62.70 mg.Kg-1; table 3.1.).

On the other hand, mean growth rate of the larvae's head was only significantly affected at 200 mg.Kg-1 (Fig. 3.5.). The mean values for growth were approximately 0.4 mm.day-1, except at the highest treatment where larvae only had a mean growth rate of 0.25 mm.day-1. An LC_{50} was extrapolated to 222.62 ± 7.71 mg.Kg-1 (Kruskal–Wallis ANOVA on Ranks, H=19.59, df=5, $p<0.05$). In the tests performed with $AgNO_3$, no significant differences were attained between the treatments used and control (data not shown), in all endpoints assessed (survival (One way ANOVA, F5, 36=1.73; $p>0.05$) head growth rate (One way ANOVA, F5, 36=2.07; $p>0.05$) and body growth rate (One way ANOVA, F5, 36=3.67; $p<0.05$; Holm-Sidak's test, $p<0.05$)).

Table 3.1. LC_{50}, EC_{50}, NOEC and LOEC (as $mg.Kg^{-1}$) values derived for the 10 days exposure of *Chironomus riparius* to AgNPs spiked sediment

Parameter	EC_{50} value	r^2	NOEC	LOEC
Survival	>200	0.65	100.00	200.00
Growth rate Body	>200	0.79	-	12.5
Growth rate Head	>200	0.89	100.00	200.00

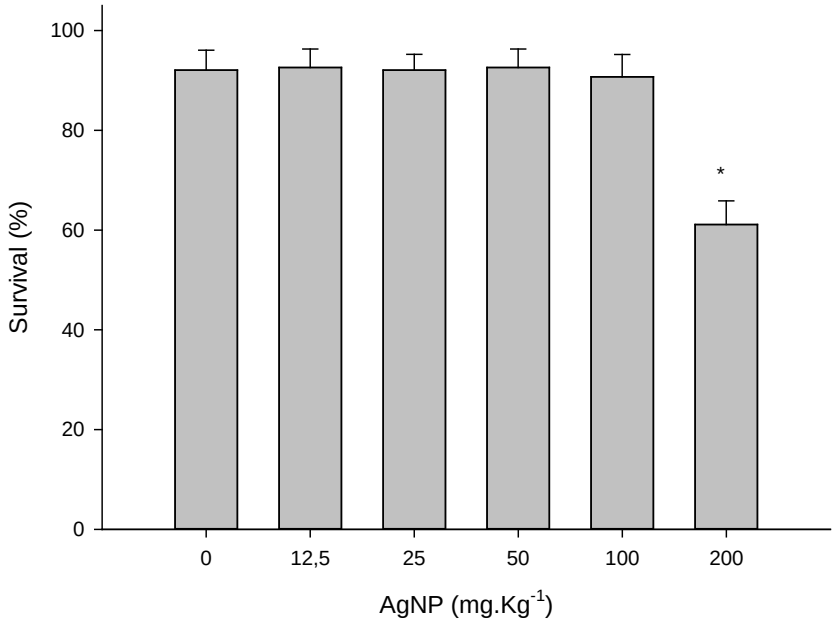

Fig. 3.3. Percentage of survival of Chironomus riparius, after a 10 day exposure to AgNPs spiked sediment. Data is expressed as mean values and standard error [(*) denotes statistical significant differences ($p<0.05$ Dunn's method)].

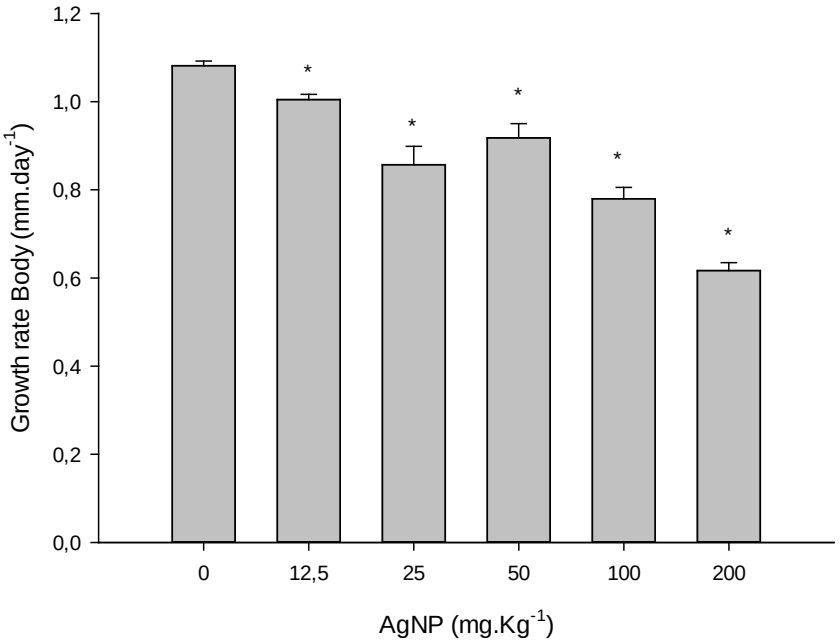

Fig. 3.4. Mean growth rate of larval body of *Chironomus riparius* expressed as mm per day, after a 10 day exposure to AgNPs spiked sediment. Data is expressed as mean values and standard error [(*) denotes statistical significant differences (p<0.05 Holm-Sidak's method)].

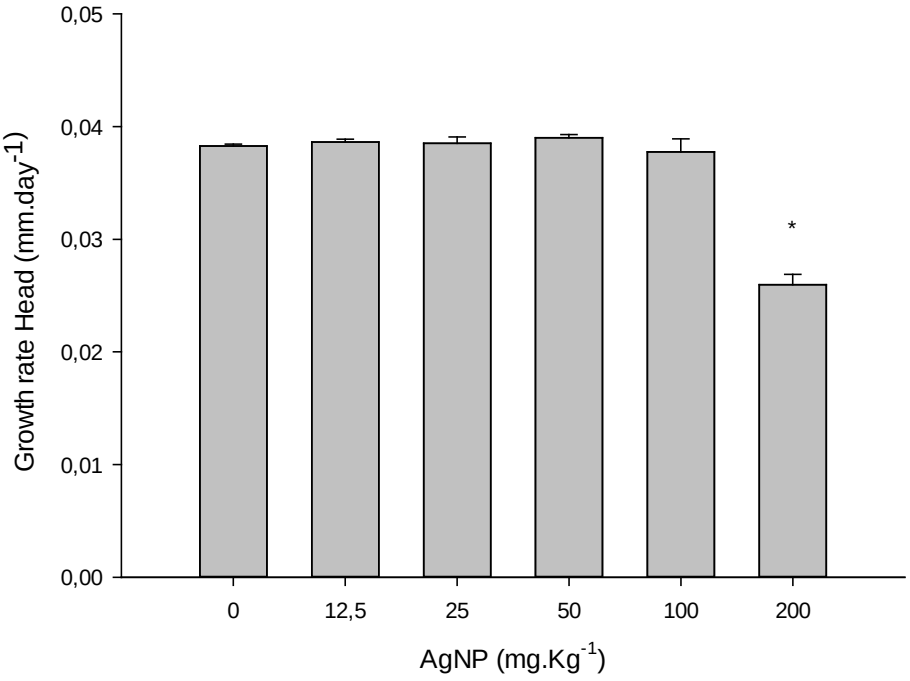

Fig. 3.5. Mean growth rate of larval head of *Chironomus riparius* expressed as mm per day, after a 10 day exposure to AgNPs spiked sediment. Data is expressed as mean values and standard error [(*) denotes statistical significant differences (p<0.05 Dunn's method)].

Mean percentage of emergence at 28 days of exposure revealed a dose response pattern (Fig. 3.6.). The two highest concentrations tested showed a significant decrease from the control, reaching only a 25% of emergence at 200 mg.Kg^{-1}. From the results a NOEC of 50 mg.Kg^{-1}, a LOEC of 100 µg.L^{-1} and an EC_{50} of 122.19 ±15.18 mg.Kg^{-1} were derived (table 3.2.) (One way ANOVA, $F_{5, 36}$=19.10; p<0.05; Holm-Sidak's test, p<0.05).

The mean number of days to emerge (Fig. 3.7.) and the adult dry weight (Fig. 3.8.) proved to be unresponsive to all AgNPs concentrations used (Kruskal–Wallis ANOVA on Ranks, H=6.19, df=5, p>0.05) for time of emergence; female weight(One way ANOVA, $F_{5, 36}$=0.49; p>0.05); and male adult weight (One way ANOVA, $F_{5, 35}$=0.19; p>0.05)along with all tests performed with $AgNO_3$, (data not shown), time to emergence(One way ANOVA, $F_{5, 36}$=2.21; p>0.05), percentage of emerged adults (One way ANOVA, $F_{5, 36}$=1.27; p>0.05), adult male weight (One way ANOVA, $F_{5, 34}$=2.10; p>0.05), and adult female weight(One way ANOVA, $F_{5, 36}$=0.45; p>0.05).

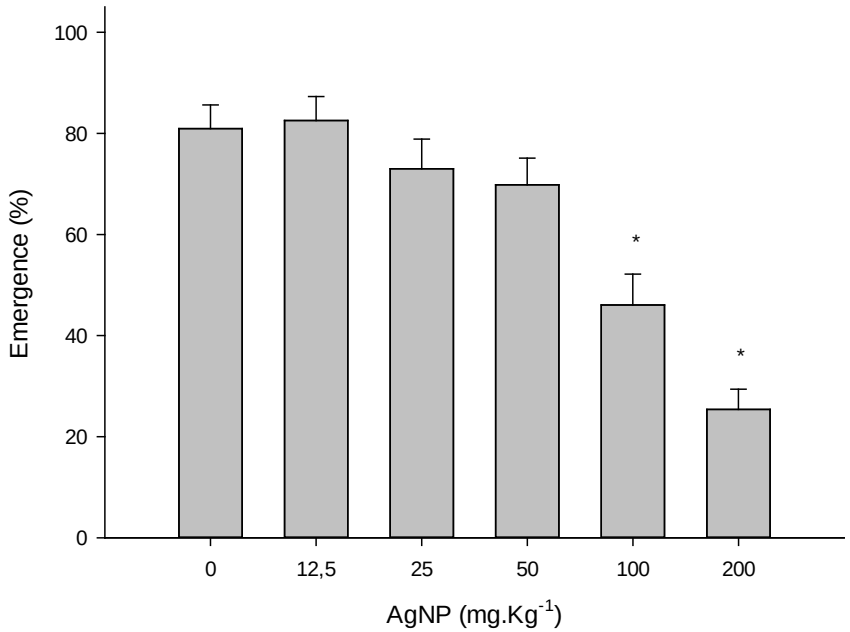

Fig. 3.6. Percentage of emergence success of *Chironomus riparius*, after a 28 day exposure to AgNPs spiked sediment. Data is expressed as mean values and standard error [(*) denotes statistical significant differences (p<0.05 Holm-Sidak's method)].

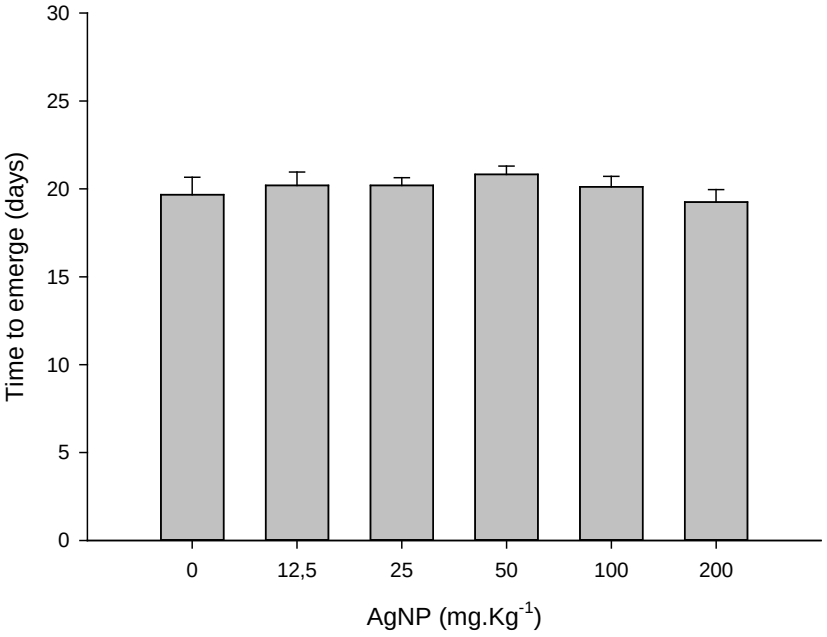

Fig. 3.7. Time to emerge of *Chironomus riparius*, after a 28 day exposure to AgNPs spiked sediment. Data is expressed as mean values and standard error.

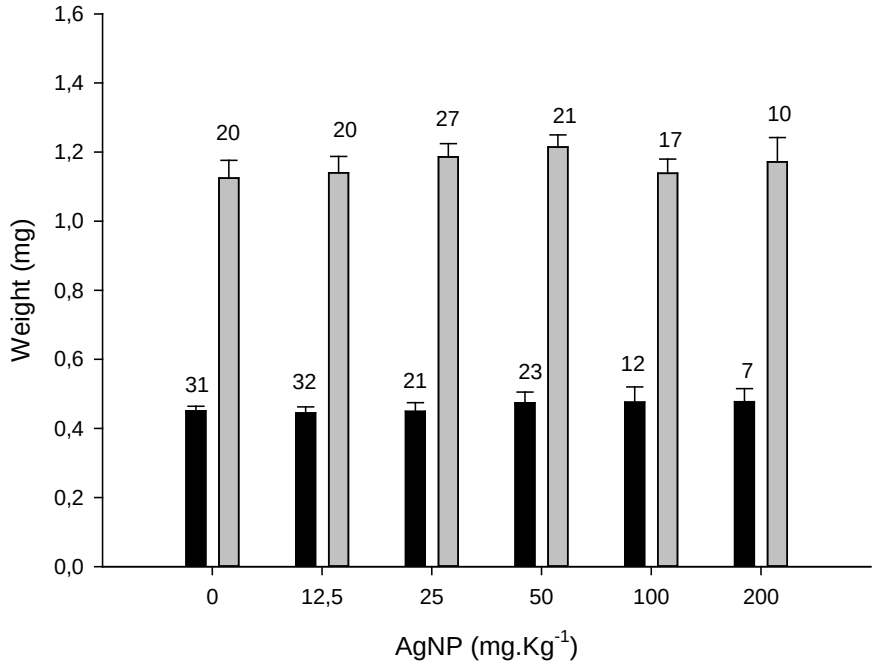

Fig. 3.8. Mean weight of male and female Chironomus riparius, after a 28 day exposure to AgNPs spiked sediment. Data is expressed as mean values and standard error. Black bars are for male weight and grey bars refer to female weight.

Table 3.2. EC_{50}, NOEC and LOEC (as $\mu g.L^{-1}$) values obtained for *Chironomus riparius* emergence, after a 28 days exposure to AgNPs spiked sediment.

Parameter	EC_{50} value	Standard error	r^2	NOEC	LOEC
Emergence	122.19	15.18	0.72	50.00	100.00

3.4.3 Biomarkers test

Organisms exposed to AgNPs spiked sediment showed a significant increase in both GST and CAT activities at the end of the 24 hours of exposure (Fig, 3.9. and Fig. 3.10., respectively). GST showed to be affected at an exposure of 50.00 mg.Kg-1 (LOEC) (One way ANOVA, $F_{3,21}$=8.47; $p<0.05$). CAT activity responded to Ag NP exposure at 100.00 mg.Kg-1 (LOEC) (. (One way ANOVA, $F_{3, 18}$=10.32; $p<0.05$;). No significant differences were observed for Ache (One way ANOVA, $F_{3, 20}$=2.35; $p>0.05$), and LPO (One way ANOVA, $F_{3, 24}$=0.39; $p>0.05$).

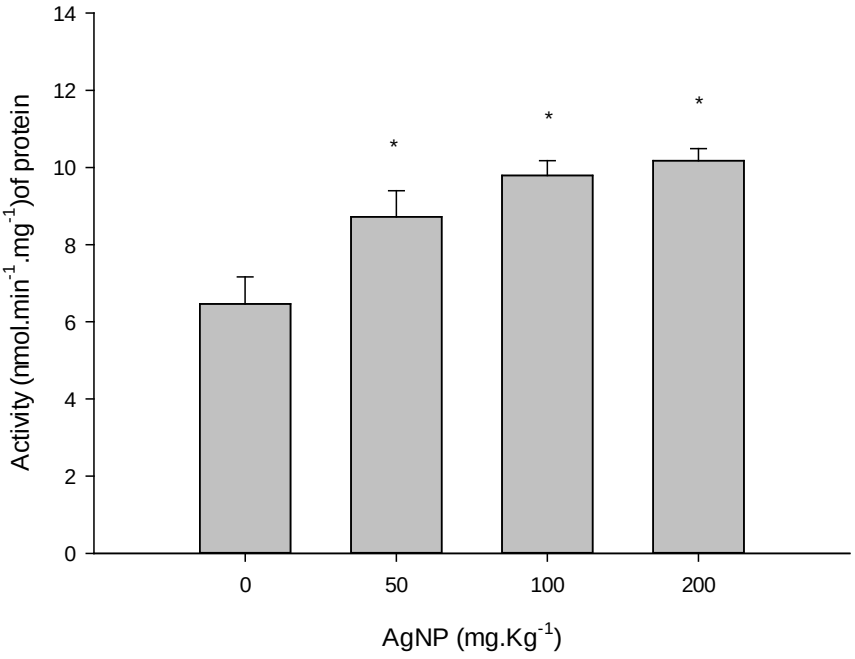

Fig. 3.9. Activity of GST of *Chironomus riparius* larvae, after a 24 hour exposure to AgNPs spiked sediment. Data is expressed as mean values and standard error [(*) Denotes statistical significant differences (p<0.05 Holm-Sidak's method)].

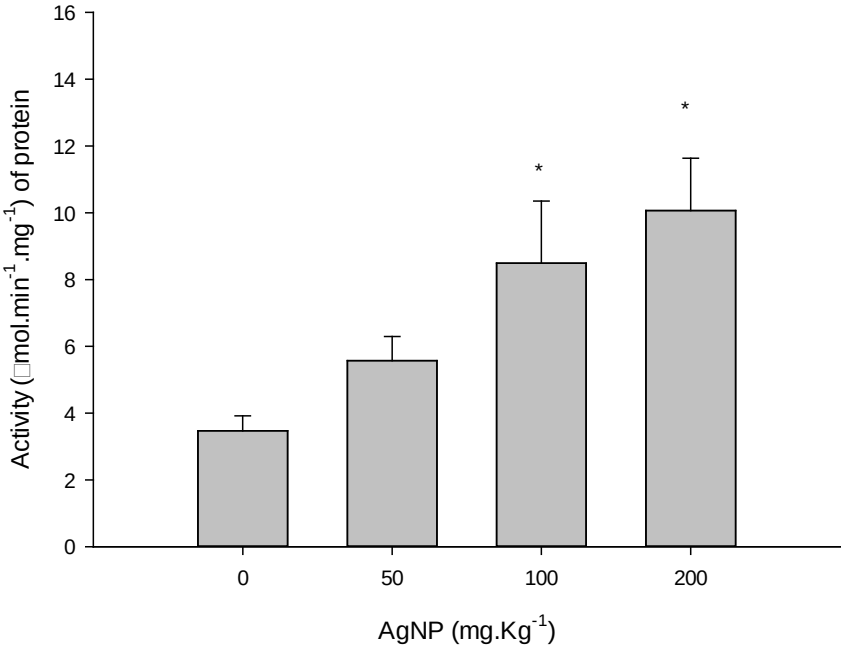

Fig. 3.10. Catalase activity of *Chironomus riparius* larvae, after a 24 hour exposure to AgNPs spiked sediment. Data is expressed as mean values and standard error [(*) Denotes statistical significant differences (p<0.05 Holm-Sidak's method)].

3.4.4 Life cycle test

Emergence success for both generations was concentration dependent (Fig. 3.11.). The values from the parental generation (F0) derived a NOEC of 25 $mg.Kg^{-1}$, LOEC of 50 $mg.Kg^{-1}$ and the EC_{50} value calculated of 75.54 ± 33.82 $mg.Kg^{-1}$ (table 3.4.) (Kruskal–Wallis ANOVA on Ranks, H=88.93, df=5, $p<0.05$). Results from the filial generation (F1) presented values of NOEC 25.00 $mg.Kg^{-1}$, LOEC 50.00 $mg.Kg^{-1}$ and the EC_{50} of 49.20 ± 0.23 $mg.Kg^{-1}$ (One way ANOVA, $F_{3, 92}$=27.31; $p<0.05$; Holm-Sidak's test, $p<0.05$).

Whilst F0 only showed slight differences in the mean day (Kruskal–Wallis ANOVA on Ranks, H=23.19, df=5, $p>0.05$), F1 demonstrated a significant reduction in the time to emerge compared to control (Fig. 3.12.), and the values obtained were 12.50 for NOEC and 25.00 for LOEC,[1] (Kruskal–Wallis ANOVA on Ranks, H=70.77, df=3, $p<0.05$). Sex ratio in controls was within the accepted validation limits (lowest 0.4, highest 0.6) and did not have major significant differences in both generations (Fig.3.13.) (Kruskal–Wallis ANOVA on Ranks, H=10.19, df=5, $p>0.05$) and (Kruskal–Wallis ANOVA on Ranks, H=0,86 df=3, $p>0.05$), for F0 and F1, respectively. Fertility of F0 was severely affected in the two highest concentrations tested (Fig. 3.15.), resulting inno live larvae hatched from the egg ropes laid from F0 females exposed to those concentrations of AgNPs. Fertility had a NOEC of 12.50 $mg.Kg^{-1}$ and LOEC of 25.00 $mg.kg^{-1}$ and a perceivable EC_{50} of 70.21 ± 8.19 $mg.Kg^{-1}$ (Table. 3.4.) (One way ANOVA, $F_{5, 6}$=85.23; $p<0.05$; Holm-Sidak's test, $p<0.05$). The endpoint of number of egg ropes per F0 females was significantly affected in regards to all treatments (Fig. 3.14.) and no NOEC was observable, with a LOEC of 25.00 $mg.Kg^{-1}$ and the calculated EC_{50} 13.10 ± 0.01 $mg.Kg^{-1}$, (Table 3.4.) (One way ANOVA, $F_{5, 6}$=9.23; $p<0.05$; Holm-Sidak's test, $p<0.05$).

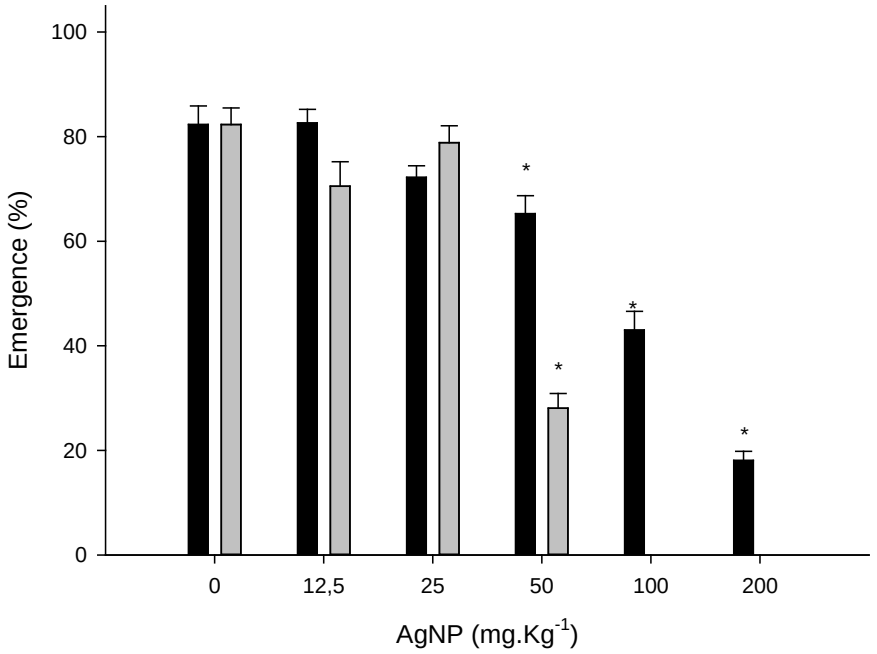

Fig. 3.11. Emergence success of individuals of *Chironomus riparius* exposed for two consecutive generations to AgNPs spiked sediment. Data is expressed as mean values and standard error, [(*) denotes statistical significant differences; for black bars (*P<0.05 Dunn's method and for grey bars (*P<0.05 Holm-Sidak's method)]; black bars are for F0's data and grey bars refer to F1.

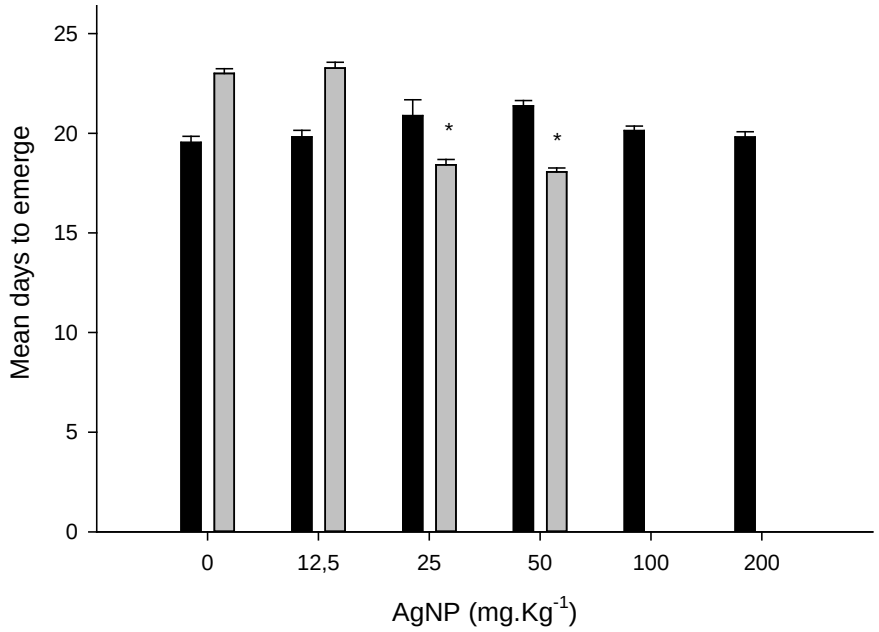

Fig. 3.12. Time to emerge of individuals of *Chironomus riparius* exposed for two consecutive generations to AgNPs spiked sediment. Data is expressed as mean values and standard error [(*) denotes statistical significant differences (p<0.05 Holm-Sidak's method)], black bars are for F0's data and grey bars refer to F1.

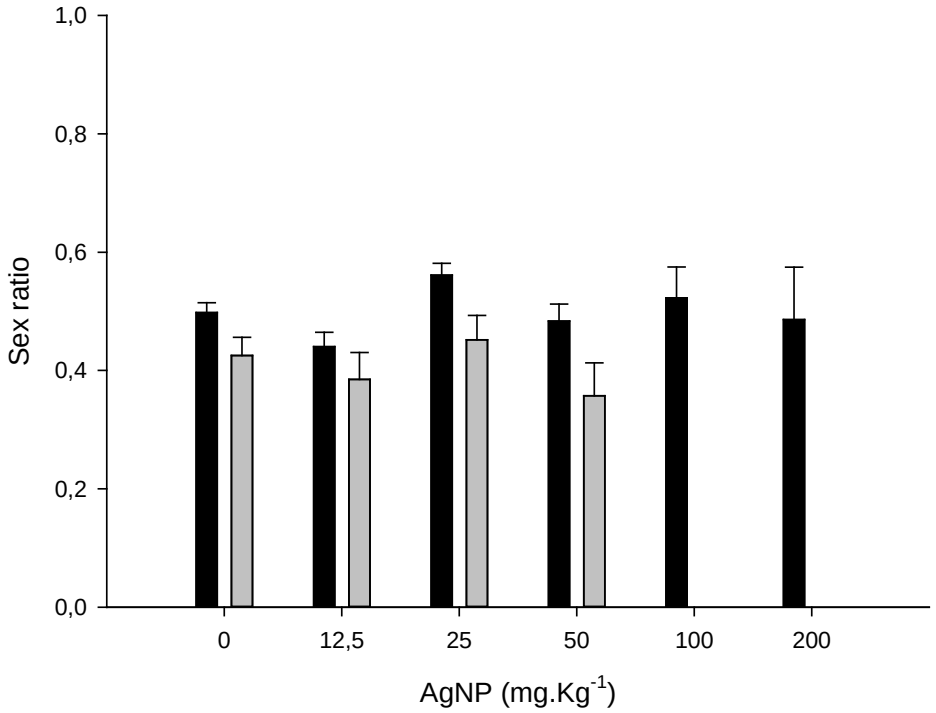

Fig. 3.13. Sex ratio at emergence of individuals of *Chironomus riparius* exposed for two consecutive generations to AgNPs spiked sediment. Data is expressed as mean values and standard error, black bars are for F0's data and grey bars refer to the F1.

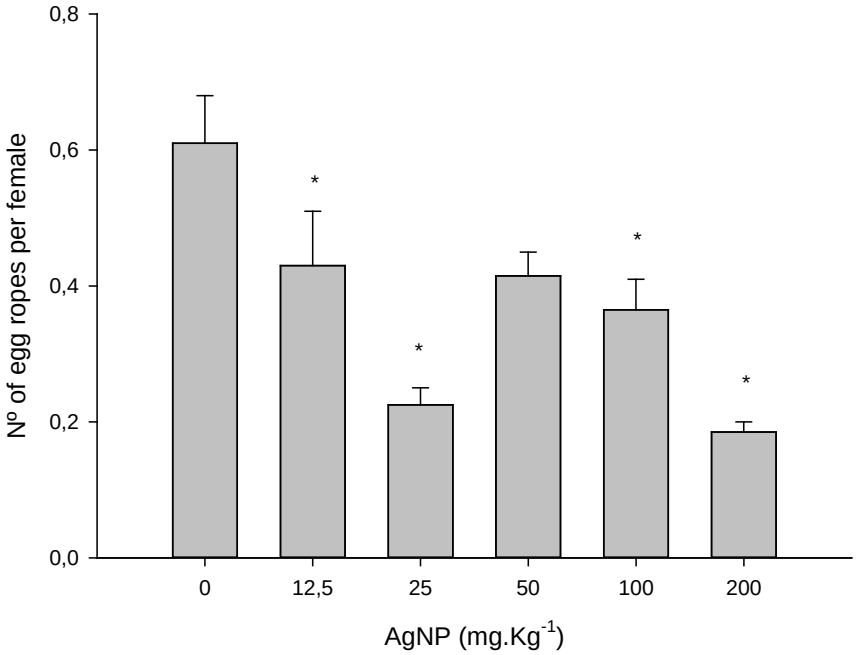

Fig. 3.14. Number of egg ropes per F0 females of *Chironomus riparius*, after exposure to AgNPs spiked sediment. Data is expressed as mean values and standard error [(*) denotes statistical significant differences ($p<0.05$ Holm-Sidak's method)].

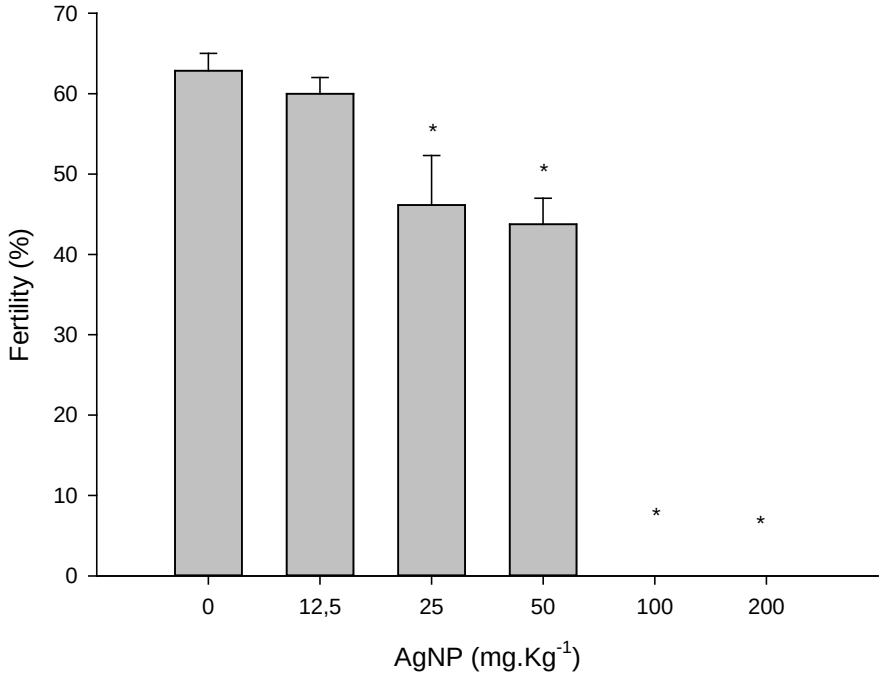

Fig. 3.15. Fertility of egg ropes of F0 of *Chironomus riparius*, after exposure to AgNPs spiked sediment. Data is expressed as mean values and standard error [(*) denotes statistical significant differences ($p<0.05$ Holm-Sidak's method)].

Table 3.3. EC_{50}, NOEC and LOEC (as $mg.Kg^{-1}$) values obtained after exposure of two consecutive generations of *Chironomus riparius* to AgNPs spiked sediment.

Parameter	EC_{50} value	Standard error	r^2	NOEC	LOEC
Emergence F0	75.54	33.82	0.74	25.00	50.00
Emergence F1	49.20	0.23	0.60	25.00	50.00
Egg ropes per female F0	13.10	0.01	0.61	-	12.50
Fertility F0	70.21	8.19	0.96	12.50	25.00

3.5 Discussion

Growth rates of head and body decreased with increasing concentrations of AgNPs. Larval body growth seemed to be a more sensitive endpoint than growth of the head capsule. In several studies similar increases in growth patterns were observed, which were correlated with increasing temperatures (Sankarperumal & Pandian 1991, Stevens 1998, Pery & Garric 2006). Likewise decreasing temperatures lead to a reduction in growth and head capsule width (Frouz et al., 2002).However, such did not occur in this experiment as temperature was stable. Head deformities in *Chironomus* have previously been reported when exposed to contaminated sediment with metals, PAHS and PCBs (Janssens de Bisthoven et al., 1998), and decreases in mean length of larvae correlated to contaminated natural sediments (Wentsel et al., 1977). Reduction of growth in individual chironomids is correlated to effects at the population level. The ability to colonize the substrate was diminished when a reduction of growth of at least 30% occurred (Giesy et al., 1988), and also a diminishing of population levels through reproduction impairment (Moore and Dillon, 1993). Silver seems to induce similar patterns of behavioral changes in other benthic organisms such as *Nereis diversicolor* (Cong et al., 2014), despite not affecting the growth of organisms (Cong et al., 2011). Survival of larvae observed is in accordance with a previous study by Call et al. (1999), which with *Chironomus tentans* found LC_{50} values for silver ranging between 1.17 and 2.75 $g.Kg^{-1}$. Larvae were also reported to have similar resistance regarding different compounds such as TBT and cadmium (Vogt et al., 2007a).

Emergence decreased both in the generations F0 and F1, corroborating the previous emergence test results. These results are also in accordance with several studies where AgNPs induced failure of emergence and pupation (Nair et al., 2011), and with similar EC_{50} values (Call et al., 1999). Time of emergence did not differ in F0, however there was a difference between controls of the two generations; with F1 presenting a dose response regarding this endpoint. Differences in emergence time between controls are under the limits accepted to this kind of life cycle (OECD, 2010). However the altered emergence patterns in F1 are indicative of populations' sensitivity when present in a contaminated

site. In aquatic insects such as chironomids differences in emergence time can lead to the disruption of reproduction and population structure (Downes, 1969; Savolainen, 1978; Colbo and Porter, 1979). Other compounds such as cadmium, tributyltin and polycyclic aromatic compounds were shown to be associated with delays in emergence and reductions of the total number of emerged individuals (Vogt et al., 2007 and Paumen et al., 2008). The decrease in emergence in the present study seems to be strongly correlated with reduction of larval growth, as also noticed in previous studies (Liber et al., 1996). Although differences in weight were correlated with differences in population fitness and deformity (Janssens de Bisthoven et al., 1998), previously reported in regards to silver (Call et al., 1999), no modifications in weight were recorded. The stable weight of adult chironomids, despite the decrease in growth rates indicates that larvae must reach a minimum weight threshold before pupation can occur. Larvae which fail to reach the minimum weight do not pupate, thus decreasing emergence rates. The existence of a minimum weight threshold for pupation has been described for *Chironomus plumosus* (Hilsenhoff, 1966) and *Chironomus tentans* (Sibley et al., 1997); and a minimum weight of 0.8mg has been suggested to be used as a validation endpoint in bioassays (Ankley et al., 1993).

The reduction of larval growth and decrease in emergence could be related to interferences with hormonal responses of ecdysones. These belong to the group of steroid hormones and are present in all arthropods (Lafont and Mathieu, 2007). Ecdysteroids play an essential role in development, growth, reproduction and embryogenesis such as: silkworm *Bombyx mori*, fruitfly *D. melanogaster* (Sekimoto et al., 2006). Ecdysteroids are targeted to the ecdysone receptor resulting in activation of downstream genes of ecdysteroid-regulated signaling pathways, this can also occur through mutations in the receptor gene (King-Jones et al., 2005) Previous studies reported that the levels of ecdysone in *C. riparius* related to receptor gene were dose dependent, when exposed to butyl-benzyl-phthalate (Herrero et al., 2015), nonylphenol AgNPs (Nair and Choi, 2012).

The number of eggs per female in F0 was variable but with a discernible dose response in relation to toxicity, however the fertility of those egg masses was strikingly affected. This has been reported in other studies with AgNPs (Nair et al., 2011) but also

similar effects were reported for TBT, cadmium, di-2-ethylhexyl phthalate (Vogt et al., 2007a; Kim and Lee, 2004). The slight increase in the number of eggs masses can be due to a response mechanism, and has been accounted before with TiO2 NPs in the silkworm insect *Bombyx mori* (Ni et al., 2015). Reproductive output was noticed to also be correlated with growth in the larval stage (Allan and Daniels, 1982).This relation seems also to be present in this study. The effects may also be from ecdysteroid hormone disruption. It was reported that any effect increasing the quantity of ecdysteroids will in *Drosophila melanogaster* affect oogenesis (Soller et al., 1999) and reproduction (Simon et al., 2003), and even inhibits regular sexual intercourse as it influences male orientation (Liu et al., 2014). Such mechanism could also act in C .riparius, explaining the results obtained in this study of the increasing infertility and the number of laid eggs per female.

The values of glutathione s-transferase activity in control (within the range of 6 $nmol.min^{-1}.mg^{-1}$ of protein) were lower than the expected and reported values for midges (20 to 54 $nmol.min^{-1}.mg^{-1}$ of protein) (Hirthe et al., 2001; Kheir et al., 2001). This may indicate that the batch used could be under a low point of resistance. However, the increase in activity of catalase and glutathione-s-transferase was concentration dependent, as shown in previous studies regarding different compounds for the 24h of exposure, reaching again similar values than in controls at 96h (Lee and Choi, 2007; Oberholster et al., 2011). The findings indicate the trigger of antioxidant defense mechanism as a consequence of the higher production of ROS, such as hydrogen peroxide which is dissociated by catalase into water and oxygen. The overall results suggest that silver nanoparticles led to pronounced effects related to degradation of oxidative stress inducing species and detoxification (Vutukuru et al., 2006; Oberholster et al., 2011; Nair, 2013) Adittionally the unresponsive nature of the LPO analysis seems to indicate lack of oxidative stress. Overall similar results of induction of catalase and glutathione s-transferase activity have been associated with genotoxicity and altered gene expression of the ribosomal protein gene (CrL15), gonadotrophin releasing hormone gene (CrGnRH1), Balbiani ring protein gene (CrBR2.2), superoxide dismutases (CuZnSOD and MnSOD). These had been reported as affecting protein synthesis, inducing reproductive failure due to disturbance of

hormonal transduction pathways and as a protection mechanism against oxidative stress (Nair et al., 2011; Nair and Choi 2011; Nair et al., 2013).

TEM imaging revealed probable presence of silver sulphide. This compound has been associated with a group of compounds which induce lower toxicity of metals (copper, iron, zinc, silver, etc.), such compounds occur through the binding of elements with ligands at the end of 48 hours (Bowles et al., 2002), chiefly in the presence of an anoxic environment (Kim et al., 2010 and Dale et al., 2013). Soluble Ag species and AgNPs are prone to bind to several ligands such as submicron particles, colloids, sulfides, thiosulfates, organic carbon and chlorides (Stumm and Morgan, 1996; Shafer et al., 1998; Wang, 2003; Kim et al., 2010). The sulfatized silver compounds will be found mostly in the sediment pore water (Rozan et al., 2000), possessing a high stability with half-life varying from 10 years to over a century (Dale et al., 2013), and therefore will have extremely low toxicity compared to free silver (LeBlanc et al., 1984). In the environment reduced sulphur occurs naturally (Kramer et al., 2007), and usually concentrations far exceed those of silver. This may explain the reason that in this study, a general lack of response and a high resistance of chironomids population was observed. Previous studies showed the same overall response, when *C. riparius* was exposed to metals and pesticides in natural waters (Pérez et al., 2010), natural sediments or in field experiments in contaminated rivers (Postma et al., 1995).

3.6 Conclusion

Sediment contaminated with AgNPs showed to be detrimental to *C. riparius*, although in extreme concentrations higher than those found in natural environments. Nevertheless, the present study provides some highlights and improvements on methodologies to understand silver toxicity.

Weight of adult midges seems not to be impaired by silver exposure.. Reproduction was impaired, shown by both the number of eggs as in their fertility, and F1 individuals are under stress as proven by an early emergence. The reduction of growth rates leads to a reduction in the biomass available for higher trophic chain organisms, this phenomenon

will also be noticeable with the increase in mortality. Populations hindered in such a way, when another stressor is introduced could lead to extinction, as evidenced also in previous studies (Ball and Baker, 1995; Van Urk et al., 1992; Peck et al., 2002; Vogt et al., 2007).

Silver nanoparticles seemed to induce processes of detoxification and prevention of oxidative stress . Further studies are needed to confirm these results and to compare with additional silver forms.

3.7 References:

ALLAN J.D. and DANIELS R.E., 1982. Life table evaluation of chronic exposure of *Eurytomora affinis* (Copepoda) to Kepone. Marine Biology, 66, 179–184.

ANKLEY G.T., BENOIT D.A., HOKE R.A., LEONARD E.N., WEST C.W., PHIPPS G.L., MATTSON V.R. and ANDERSON L.A., 1993. Development and evaluation of test methods for benthic invertebrates and sediments: Effects of flow rates and feeding regime on water quality and exposure conditions. Archives of Environmental Contamination and Toxicology, 25, 12–19.

BALL S.L. and BAKER R.L., 1995. The non-lethal effects of predators and the influence of food availability on the life history of adult *Chironomus tentans* (Diptera: Chironomidae). Freshwater Biology, 34, 1–12.

BBC RESEARCH, 2014. Global markets for nanocomposites, nanoparticles, nanoclays and nanotubes. 2014 Nanotechnology Research Review, Report Code: NAN047F; Business Communications Company, December 2014.

BIRD R.P. and DRAPER H.H., 1984. COMPARATIVE STUDIES ON DIFFERENT METHODS OF MALONALDEHYDE DETERMINATION. METHODS IN ENZYMOLOGY. 105, 299 – 305.BURTON G.A.JR., 1991. Assessing the toxicity of freshwater sediments. Environmental Toxicology and Chemistry, 10, 1585-1627.

BOWLES K.C., BIANCHINI A., BRAUNER C.J., KRAMER J.R. and WOOD C.M., 2002. Evaluation of the effect of reactive sulfide on the acute toxicity of silver (I) to Daphnia magna. Part 1: Description of the chemical system. Environmental Toxicology and Chemistry, 21, 1286–1293.

CALL D.J., POLKINGHORNE C.N., MARKEE T.P., BROOKE L.T., GEIGER D.L., GORSUCH J.W. and ROBILLARD K.A., 1999. Silver Toxicity to Chironomus Tentans in Two Freshwater Sediments. Environmental Toxicology and Chemistry, 18(1), 30–39.

CLAIRBORNE A., 1985. Catalase activity. In: Greenwald, R.A. (Ed.), CRC Handbook of Methods in Oxygen Radical Research, pp. 283-284. CRC Press, Boca Raton, FL.

COLBO M.H. and PORTER G.N., 1979. Effects of food supply on the life history of Simuliidae (Diptera). Canadian Journal of Zoology, 57, 301–306.

CONG Y., BANTA G.T., SELCK H., BERNAHU D., VALSAMI-JONES E. and FORBES V.E., 2011. Toxic effects of bioaccumaltion of nano-, micron- and ionic-Ag in the polychate *Nereis diversicolor*. Aquatic Toxicology 105, 403-411.

CONG Y., BANTA G.T., SELCK H., BERHANU D., VALSAMI-JONES E. and FORBES V.E., 2014. Toxicity and bioaccumulation of sediment-associated silver nanoparticles in the estuarine polychaete*, Nereis (Hediste) diversicolor*. Aquatic Toxicology, 156, 106–115.

DALE A.L., LOWRY G.V. and CASMAN E.A., 2013. Modeling Nanosilver Transformations in Freshwater Sediments. Environmental Science & Technology, 47, 12920 −12928.

DAVID C.P., 2003. Establishing the Impact of Acid Mine Drainage through Metal Bioaccumulation and Taxa Richness of Benthic Insects in a tropical Asian Stream (The Philippines). Environmental Toxicology and Chemistry, (22)12, 2952-2959.

DAY K. and KAUSHIK N.K., 1987. An assessment of the chronic toxicity of the synthetic pyrethroid, fenvalerate, to *Daphnia galeata mendotea,* using life tables. Environmental Pollution, 44, 13–26.

DE HAAS E. M., WAGNER C., KOELMANS A.A., KRAAK M.H.S. and ADMIRAAL W., 2006. Habitat selection by chironomid larvae: fast growth requires fast food. Journal of Animal Ecology, 75(1), 148-155.

DOBIAS J. and BERNIER-LATMANI R., 2013. Silver Release from Silver Nanoparticles in Natural Waters. Environmental Science & Technology, 47, 4140-4146.

DOWNES J.A., 1969. The swarming and mating flight of Diptera. Annual Review Entomology, 14, 271–298.

DYAR H.G., 1890. The number of molts of lepidopterous larvae. Psyche, 5, 420 – 422.

ELLMAN G.L., COURTNEY K.D.; ANDRES V.JR. and FEATHERSTONE R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, 7, 88-95.

EPA U.S. Environmental Protection Agency and Army Corps of Engineers. 1991. Ecological evaluation of dredged material proposed for ocean disposal-testing manual. EPA 503/8-91-001. Washington, DC.

EPA U.S. Environmental Protection Agency and Army Corps of Engineers. 1993. Draft ecological evaluation of dredged material proposed for discharge to inland and near-coastal waters—Testing manual. EPA 503/8-91-001. Washington, DC.

FROUZ J., ALI A. and LOBINSKE R.J., 2002. Influence of temperature on developmental rate, wing length, and larval head capsule size of pestiferous midge *Chironomus crassicaudatus* (Diptera: Chironomidae). Journal of Economic Entomology, 95, 699–705.

GABRIELS W., LOCK K., DE PAUW N. and GOETHALS P.L.M., 2010. Multimetric macroinvertebrate index flanders (MMIF) for biological assessment of rivers and lakes in Flanders (Belgium). Limnologica, 40, 199−207.

GIESY J.P., GRANEY R.L., NEWSTEAD J.L., ROSIU C.J., BENDA A., KREIS R.G.JR. and HORVATH F.J., 1988. Comparison of three sediment bioassay methods using Detroit River sediments. Environmental Toxicology and Chemistry, 7, 483–498.

GUILHERMINO L., LOPES M.C., CARVALHO A.P. and SOARES A.M.V.M., 1996. Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia magna*. Chemosphere, 32: 727:738

HABIG W., PABST M.J. and JAKOBY W.B., 1974. Glutathione s-transferases - The first enzymatic step in mercapturic acid formation. The Journal of Biological Chemistry, 249, 22, 7130-7139.

HANDY R.D., VAN DEN BRINK N., CHAPPELL M., MÜHLING M., BEHRA R., DUSINSKA M., SIMPSON P., AHTIAINEN J., JHA A.N., SEITER J., BEDNAR A., KENNEDY A., FERNANDES T.F. and RIEDIKER M., 2012. Practical considerations for conducting ecotoxicity test methods with manufactured nanomaterials: what have we learnt so far? Ecotoxicology, 21,933–972.

HERRERO O., PLANELLÓ P. and MORCILLO G., 2015. The plasticizer benzyl butyl phthalate (BBP) alters the ecdysone hormone pathway, the cellular response to stress, the energy metabolism, and several detoxication mechanisms in *Chironomus riparius* larvae. Chemosphere**,** 128, 266 – 277.

HILSENHOFF W.L., 1966. The biology of *Chironomus plumosus* (Diptera: Chironomidae) in Lake Winnebago, Wisconsin. Annals of the Entomological Society of America, 59, 365–473.

HIRTHE G., FISHER T.C., CRANE M. and CALLAGHAN A., 2001. Short-term exposure to sub-lethal doses of lindane affects developmental parameters in *Chironomus riparius* Meigen, but has no effect on larval glutathione-S-transferase activity. Chemosphere 44, 583–589.

JANSSENS DE BISTHOVEN L., NUYTS P., GODDEERIS B. and OLLEVIER F., 1998. Sublethal parameters in morphologically deformed *Chironomus* larvae: Clues to understanding their bioindicator value. Freshwater Biology, 39, 179–191.

KHEIR R., IBRAHIM H., LEWIS J., CALLAGHAN A. and CRANE M., 2001. Comparison of acetylcholinesterase and glutathione S-transferase activity in *Chironomus riparius* Meigen exposed to chemical-spiked sediments. B. Environmental Contamination and Toxicology. 66, 603–610.

KIM E.J. and LEE S.K., 2004. Reduced viability of F1 egg ropes in Chironomus riparius exposed to di-2-ethylhexyl phthalate (DEHP). Journal of Environmental Biology, 25(3), 259-261.

KIM B., PARK C., MURAYAMA M. and HOCHELLA M.F.JR., 2010. Discovery and Characterization of Silver Sulfide Nanoparticles in Final Sewage Sludge Products. Environmental Science & Technology, 44, 7509–7514.

KING-JONES K., CHARLES J., LAM G. and THUMMEL C.S., 2005. The Ecdysone-Induced DHR4 Orphan Nuclear Receptor Coordinates Growth and Maturation in Drosophila. Cell, 121, 773 – 784.

KLAINE S.J., ALVAREZ P.J.J., BATLEY G.E., FERNANDES T.F., HANDY R.D. and LYON, D.Y., 2008. Nanomaterials in the environment: behaviour, fate, bioavailability, and effects. Environmental Toxicology and Chemistry, 27, 1825–1851.

KOSALWAT P. and KNIGHT A.W., 1987. Chronic Toxicity of Copper to a Partial Life Cycle of the Midge, Chironomus decorus. Archives Environmental Contamination and Toxicology, 16, 283-290.

KRAMER J.R., BELL R.A. and SMITH D.S., (2007). Determination of sulfide ligands and association with natural organic matter. Appllied Geochemistry, 22, 1606–11.

LAFONT, R. and MATHIEU, M., 2007. Steroids in aquatic invertebrates. Ecotoxicology, 16, 109 –130.

LEAL H., SOETER A.M., KOOLS S.A.E., KRAAK M.H.S., PARSONS J.R., TEMMINK H., ZEEMAN G. and BUISMAN C.J.N., 2012. Ecotoxicological assessment of grey water treatment systems with Daphnia magna and Chironomus riparius. Water Research, 46, 1038-1044.

LEBLANC G.A., MASTONE J.D., PARADICE A.P., WILSON B.F., LOCKHART H.B.JR. and ROBILLARD K.A., 1984. The influence of speciation on the toxicity of silver to fathead minnow (*Pimephalies promelas*). Environmental Toxicology and Chemistry, 3, 37–47.

LEE S.B. and CHOI J., 2007. Effects of bisphenol A and ethynylestradiol exposure on enzyme activities, growth and development in the four instar larvae of *Chironomus riparius* (Diptera, Chironomidae). Ecotoxicoly and Environmental Safety, 68, 84–90.

LIBER K., CALL D.J., DAWSON T.D., WHITEMAN F.W. and DILLON T.M., 1996. Effects of *Chironomus tentans* larval growth retardation on adult emergence and ovipositing success: Implications for interpreting freshwater sediment bioassays. Hydrobiologia, 323, 155–167.

Liu T., Li Y., Zhao X., Zhang M. and Gu W., 2014. Ethylparaben affects lifespan, fecundity, and the expression levels of ERR, EcR and YPR in Drosophila melanogaster. Journal of Insect Physiology, 71, 1–7.

LUOMA S.N., 2008. Silver Nanotechnologies and the Environment: Old Problems or New Challenges? Project on Emerging Nanotechnologies. Woodrow Wilson Centre, Washington, DC, USA.

MARINKOVIC, M., DE BRUIJN K., ASSELMAN M., BOGAERT M., JONKER M. J., KRAAK M. H. and ADMIRAAL W., 2012. Response of the nonbiting midge Chironomus riparius to multigeneration toxicant exposure. Environmental Science & Technology, 46(21), 12105-12111.

MILLER M.P. and HENDRICKS A. C., 1996. Zinc resistance in Chironomus riparius: Evidence for physiological and genetic components. Journal of the North American Benthological Society, 15, 106−116.

MOORE D.W. and DILLON T.M., 1993. The relationship between growth and reproduction in the marine polychaete *Neries* (*Neanthes*) *arenaceodantata* (Moore): Implications for chronic sublethal bioassays. Journal of the Experimental Marine Biology and Ecology, 173, 231–246.

MORGAN A.J., KILLE P. and STÜRZENBAUM S.R., 2007. Microevolution and ecotoxicology of metals in invertebrates. Environmental Science & Technology, 41, 1085−1096.

NAIR P.M.G. and CHOI J., 2011. Identification, characterization and expression profiles of Chironomus riparius glutathione S-transferase (GST) genes in response to cadmium and silver nanoparticles exposure. Aquatic Toxicology, 101, 550–560.

NAIR P.M.G., PARK S.Y., LEE S.W. and CHOI J., 2011. Differential expression of ribosomal protein gene, gonadotrophin releasing hormone gene and Balbiani ring protein gene in silver nanoparticles exposed Chironomus riparius. Aquatic Toxicology, 101, 31–37.

NAIR P.M.G., PARK S.Y. and CHOI J., 2013. Evaluation of the effect of silver nanoparticles and silver ions using stress responsive gene expression in Chironomus riparius. Chemosphere, 92, 592–599.

NANOSILVER SUSPENSION AX 06HX, AMEPOX http://www.amepox-mc.com/cache/files/1298757566_NanoSilver_Suspension_AX_06Hx.pdf Accessed March 2015.

NI M., LI F., WANG B., XU K., ZHANG H., HU J., TIAN J., SHEN W. & LI B., 2015. Effect of TiO2 Nanoparticles on the Reproduction of Silkworm. Biological Trace Elements Research, 164, 106-113.

NOWACK B. and BUCHELI T.D., 2007. Occurrence, behavior and effects of nanoparticles in the environment. Environmental Pollution, 150, 5-22.

OBERHOLSTER P. J., MUSEE N., BOTHA A.M., CHELULE P.K., FOCKE W.W. and ASHTON P.P., 2011. Assessment of the effect of nanomaterials on sediment-dwelling invertebrate *Chironomus tentans* larvae. Ecotoxicological and Environmental Safety, 74(3), 416-423.

OECD, 2004. Test guideline 219. Sediment-water chironomid toxicity test using spiked water. OECD Guidelines for the testing of chemicals.

OECD, 2010. Test guideline 233. Sediment-water chironomid life-cycle toxicity test using spiked water or spiked sediment. OECD Guidelines for the testing of chemicals.

OECD, 2011. Test guideline 235. Chironomus sp., acute immobilisation test. OECD Guidelines for the testing of chemicals.

OZKAN N. and CAMUR-ELIPEK B., 2007: Relationships between chironomid communities (Diptera:Chironomidae) and environmental parameters in Sazlidere stream (Turkish Thrace). Tiscia, 36, 29-34.

PARK E.J., YI J., KIM Y., CHOI K. and PARK K., 2010. Silver nanoparticles induce cytotoxicity by a Trojan-horse type mechanism. Toxicology in Vitro, 24, 872–878.

PAUMEN M.L., BORGMAN E., KRAAK M.H.S., VAN GESTEL C.A.M. and ADMIRAAL W., 2008. Life cycle responses of the midge Chironomus riparius to polycyclic aromatic compound exposure. Environmental Pollution, 152, 225-232.

PECK M.R., KLESSA D.A. and BAIRD D.J., 2002. A tropical sediment toxicity test using the dipteran Chironomus crassiforceps to test metal bioavailability with sediment pH change in tropical acid-sulfate sediments. Environmetal Toxicology and Chemistry, 21(4), 720–728.

PERY A.R.R. and GARRIC J., 2006. Modelling effects of temperature and feeding level on the life cycle of the midge *Chironomus riparius*: an energy based modelling approach. Hydrobiologia, 553, 59–66.

PÉREZ J.R., LOUREIRO S., MENEZES S., PALMA P., FERNANDES R.M., BARBOSA I.R. and SOARES A.M.V.M, 2010. Assessment of water quality in the Alqueva Reservoir (Portugal) using bioassays. Environ Sci Pollut Res, 17, 688-702.

POSTMA, J.F. and C. DAVIDS. 1995. Tolerance induction and life cycle changes in cadmium-exposed *Chironomus riparius* (Diptera) during consecutive generations. Ecotoxicological and Environmental Safety, 30, 195–202.

POSTMA J.F., VAN NUGTEREN P. and BUCKERT-DE JONG M.B. 1996. Increased Cadmium Excretion in Metal-adapted Populations of the Midge *Chironomus riparius* (Diptera). Environmental Toxicology and Chemistry, 15(3), 332–339.

RIBEIRO R., KELLY L.A., GONÇALVES F., BURTON G.A.JR. & SOARES A.M.V.M., 1999. New Artificial Sediment for *Chironomus riparius.* Bulletin of Environmental Contamination and Toxicology. 63, 691— 697.

RODRIGUES A.C.M., GRAVATO C., QUINTANEIRO C., GOLOVKO O., ŽLÁBEK V., BARATA C., SOARES A.M.V.M and Pestana J.L.T, 2015. Life history and biochemical effects

of chlorantraniliprole on *Chironomus riparius*. Science of the Total Environment, 508,506–513.

ROSIU C.J., GIESY J.P. and KRIES R.G.JR., 1989. Toxicity of vertical sediments in the Trenton Channel, Detroit River, Michigan, to *Chironomus tentans* (Insecta: Chironomidae). Journal of Great Lakes Research, 15, 570–580.

ROZAN T.F., LASSMAN M.E., RIDGE D.P. and LUTHER G.W., 2000. Evidence for iron, copper and zinc complexation as multinuclear sulphide clusters in oxic rivers. Nature, 406, 879–82.

SANKARPERUMAL G. and PANDIAN T.J., 1991. Effect of temperature and chlorella density on growth and metamorphosis of *Chironomus circumdatus* (Kieffer) (Diptera). Aquatic Insects, 13, 167–177.

SAVOLAINEN E., 1978. Swarming in Ephemeroptera: the mechanism of swarming and the effects of illumination and weather. In *Annales Zoologici Fennici* (pp. 17-52). Finnish Academy of Sciences, Societas Scientiarum Fennica, Societas pro Fauna et Flora Fennica and Societas Biologica Fennica Vanamo.

SCOTT-FORDSMAND J.J., KROGH P.H., SCHAEFER M. and JOHANSEN A., 2008. The toxicity testing of double-walled nanotubes-contaminated food to *Eisenia veneta* earth-worms. Ecotoxicological and Environmental Safety, 71, 616–619.

SEKIMOTO, T., IWAMI, M. and SAKURAI, S., 2006. Coordinate responses of transcription factors to ecdysone during programmed cell death in the anterior silk gland of the silk worm, *Bombyx mori*. Insect Mol. Biol. 15, 281–292.

SHAFER M.M., OVERDIER J.T. and ARMSTONG D.E., 1998. Removal, partitioning, and fate of silver and other metals in wastewater treatment plants and effluent-receiving streams. Environmental Toxicology and Chemistry, 17, 630–641.

SIBLEY P.K., BENOIT D.A. and ANKLEY G.T., 1997. The significance of growth in *Chironomus tentans* sediment toxicity tests: Relationship to reproduction and demographic endpoints. Environmental Toxicology and Chemistry 16 (2): 336-345STEVENS

M.M., 1998. Development and survival of *Chironomus tepperi* Skuse (Diptera: Chironomidae) at a range of constant temperatures. Aquatic Insects, 20, 181–188.

SIMON A.F., SHIH C., MACK A. and BENZER S., 2003. Steroid Control ofLongevity in *Drosophila melanogaster.* Science, 28: 5611, 1407-1410.

STRELOKE M. and KÖPP H., 1995. Long-term toxicity testwith Chironomus riparius: Development and validation of a new test system. Berlim, Blackwell Wissenschafts-Verlag.(Mitteilungen aus der Biologischen Bundesansfalt für Land-und Forstwirtschaft Berlin-Dahlem; H.315) ISBN 3-8263-3077-3.

STRINI A., CASSESE S. and SCHIAVI L., 2005. Measurement of benzene, tolulene, ethelbenzene and o.xylene gas phase photodegradation by titanium dioxide dispersed in cemetitious material using a mixed flow reactor. Apllied Catalalysis, B 61 90-97.

STUMM W. and MORGAN J.J. Aquatic Chemistry. Chemical equilibria and rates in natural waters. Third edition. John Wiley & Sons, Inc; 1996. 284 p.

SNELL T.W. and HICKS D.G., 2009. Assessing Toxicity of Nanoparticles Using *Brachionus manjavacas* (Rotifera). Environmental Toxicology, 26(2), 146-152

SOLLER, M., BOWNES, M. and KUBLI, E., 1999. Control of oocyte maturation in sexually mature Drosophila females. Developmental Biology, 208, 337 – 351.

SPSS, 2008. SigmaPlot for Windows (version 11.0). Science California, USA.

TIMMERMAN K.R. and WALKER P.A., 1989. The Fate of Trace Metals During the Metamorphosis of Chironomids (Diptera: Chironomidae). Environmental Pollution, 62, 73-85.

VAN CAPPELLEN P. and WANG Y., 1995. Metal cycling in surface sediments: modeling the interplay of transport and reaction. In Allen HE, ed, *Metal Contaminated Aquatic Sediments.* Ann Arbor Press, Ann Arbor, MI, USA, pp 2–64.

VAN URK G., Kerkum F.C.M. and Smit H., 1992. Life cycle patterns, density, and frequency of deformities in *Chironomus* larvae (Diptera: Chironomidae) over a

contaminated sediment gradient. Canadian Journal of Fisheries and Aquatic Sciences, 49, 2291–2299.

VEDAMANIKAM V.J. and SHAZILLI N.A.M., 2008. The Effect of Multi-generational Exposure to Metals and Resultant Change in Median Lethal Toxicity Tests Values over Subsequent Generations. Bulletin Environmental Contamination Toxicology, 80, 63–67.

VOGT C., BELZ D., GALLUBA S., NOWAK C., OETKEN M. and OEHLMANN J., 2007a. Effects of cadmium and tributyltin on development and reproduction of the non-biting midge *Chironomus riparius* (Diptera)—baseline experiments for future multi-generation studies. *Journal of Environmental Science and Health Part A,* 42, 1–9.

VOGT C., PUPP A., NOWAK C., JAGODZINSKI L.S., BAUMMANN J., JOST D., OETKEN M. and OEHLMANN J., 2007. Interaction between genetic diversity and temperature stress on life-cycle parameters and genetic variability in midge *Chironomus riparius* populations. Climate Research, 33, 207-214.

VUTUKURU S.S., CHINTADA S., MADHAVI K.R., RAO J.V. and ANJANEYULU Y., 2006. Acute effects of copper on superoxidedismutase, catalase and lipid peroxidation in the freshwater teleost fish, *Esomus danricus*. Fish Physiology and Biochemistry, 32, 221–229.

WATTS M.M. and PASCOE D., 2000. A comparative study of *Chironomus riparius* Meigen and *Chironomus tentans* Fabricius (Diptera:Chironomidae) in aquatic toxicity tests. Archives of Environmental Contamination and Toxicology, 39, 299–306.

WALTERS C.R., POOL E.J. and SOMERSET V.S., 2014. Ecotoxicity of silver nanomaterials in the aquatic environment: a review of literature and gaps in nano-toxicological research. Journal of Environmental Science and Health Part A, 49(13), 1588-1601.

WANG J.M., 2003. Interactions of silver with wastewater constituents. Water Research, 37, 4444–4452.

WENTSEL R., MCINTOSH A. and ATCHISON G., 1977. Sublethal effects of heavy metal contaminated sediment on midge larvae (*Chironomus tentans*). Hydrobiologia, 56, 153–197.

Chapter 4

General discussion and conclusion

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4.1 General discussion and conclusion

The findings in this study suggest that egg masses are one of the most resilient stages, which may be correlated to the external gelatinous matrix that acts as a barrier for chemicals (Kosalwat and Knight, 1987). In contrast the first instar was one of the most sensitive stages to aquatic contamination with $AgNO_3$ and AgNP. In these stages no counterpart acute test for toxicity in sediment was performed, as eggs and first larval stage chironomidae within the first 48 hours mainly subsist in the water column. The high sensitivity of juvenile organisms has been previously stated and results express across several species (García-Alonso et al., 2014). Additionally, from the second to the fourth larval instar there appears to be an increase in resilience. These results are in accordance with previous studies (Pascoe et al., 1989).

Despite no toxicity being observed in spiked water, contaminated sediment had detrimental effects in growth rates and survival at the end of 10 days. This may be a consequence of the constant exposure of larvae to the sediment after the 1^{st} instar. Exposure to AgNPs in water led to a delay of emergence. Interestingly sediment toxicity only induced early emergence in the second generation of chironomids and no effect-concentration related phenomena were noticeable in the first generation. Weight in adults was not affected by AgNPs, neither through aquatic or sediment exposure. This may be correlated to a minimum weight at start of pupation, as previously reported in chapter 3. Fertility decreased in contaminated sediment, which can indicate that subsequent generation may be moving towards extinction. Further studies are needed to assert this hypothesis.

Chronic toxicity in water seemed to be more elusive to assess than toxicity in sediment. AgNPs when present in the aquatic medium undergo complex interactions which may lead to a mere transitory state in aquatic systems. Furthermore in this study silver ions showed to be more toxic than silver nanoparticles in water, although no comparisons could

be crafted in regards to chronical toxicity, as no EC_{50} value was retrieved for $AgNO_3$. Nevertheless, it is worth noting that unanimity around the higher toxicity of silver ions or AgNPs is still not clear (Kennedy et al., 2010; Cong et al., 2011; Kwok et al., 2012; Edmond, 2014). This ambiguity may be explained by the different types of NPs used, the binding capacity of media and other processes that AgNPs undergo when exposed to the test media or natural aquatic environments. There is a general consensus that further studies on the characterization of the transformation products of AgNPs are needed (Levard et al., 2012).

Research on the effects on NPs are still in its infancy especially in the sediment, additionally further investigations are needed to safely acknowledge the potential effects in the environment and human health (Dos Santos et al., 2014).

4.2 Dissemination of Results

The results obtained in the present work regarding, egg masses responses, acute toxicity and chronic toxicity both in contaminated water and sediment, were exhibited and presented at SETAC Europe 24th Annual Meeting held in Basel, Switzerland, under the name "Response of *Chironomus riparius* to silver ions and silver nanoparticles".

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4.4 References

CONG Y., BANTA G.T., SELCK H., BERNAHU D., VALSAMI-JONES E. and FORBES V.E., 2011. Toxic effects of bioaccumulation of nano-, micron- and ionic-Ag in the polychate *Nereis diversicolor*. Aquatic Toxicology 105, 403-411.

DOS SANTOS C.A., SECKLER M.M; INGLE A.P., GUPTA I., GALDIERO S., GALDIERO M., GADE A. and RAIZ M.,2014. Silver Nanoparticles: Therapeutical Uses, Toxicity, and Safety Issues. Journal of Pharmaceutical Sciences, 103, 1931–1944.

EDMOND C. Toxicokinetics and interaction of nanoparticles within biological matrices in Nanotechnology and human health. Boca Raton: CRC Press, 2014 ISBN 13:978-0-8493-8144-7. Chap. 9, p. 165-177

GARCÍA-ALONSO J., RODRIGUEZ-SANCHEZ N., MISRA S.K., VALSAMI-JONES E., CROTEAU M., LUOMA S.N. and RAINBOW P.S., 2014. Toxicity and accumulation of silver nanoparticles during development of the marine polychaete *Platynereis dumerilii*. Science of the Total Environment, 476–477, 688–695

KENNEDY A.J., HULL M., BEDNAR A.J., GOSS J.D., GUNTER J., BOULDIN J.R.L, VIKESLAND P.R.J. and STEEVENS J., 2010. Fractionating Nanosilver: Importance for Determining Toxicity to Aquatic Test Organisms. Environmental Science & Technology, 44, 9571–9577.

KOSALWAT P. and KNIGHT A.W. (1987) Chronic Toxicity of Copper to a Partial Life Cycle of the Midge, Chironomus decorus. Archives of Environmental Contamination and Toxicology, 16, 283-290.

KWOK K.W.H., AUFFAN M., BADIREDDY A.R., NELSON C.M., WIESNER M.R., CHILKOTI A.,LIU J., MARINAKOS S.M. and HINTON D.E., 2012. Uptake of silver nanoparticles and toxicity to early life stages of Japanese medaka (*Oryzias latipes*): Effect of coating materials. Aquatic Toxicology, 120– 121, 59– 66.

LEVARD C., HOTZE E. M., LOWRY G. V. and BROWN G. E.JR., 2012. Environmental transformations of silver nanoparticles: impact on stability and toxicity. Environmental Science & Technology, 46 (13), 6900-6914.

PASCOE D., WILLIAMS K.A. and GREEN D.W.J., 1989. Chronic toxicity of cadmium to *Chironomus riparius* Meigen—Effects upon larval development and adult emergence. Hydrobiologia, 175, 109–115.