Universidade do Minho
Escola de Ciências

Daniela Miranda Batista

Impacts of Silver Nanoparticles in Freshwater Detrital Food-Webs in a Warming Scenario

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Impacts of Silver Nanoparticles in Freshwater Detrital Food-Webs in a Warming Scenario

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Trabalho efetuado sob a orientação da
Professora Doutora Fernanda Cássio
e da
Professora Doutora Cláudia Pascoal

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DECLARAÇÃO DE INTEGRIDADE

Declaro ter atuado com integridade na elaboração da presente tese. Confirmo que em todo o trabalho conducente à sua elaboração não recorri à prática de plágio ou a qualquer forma de falsificação de resultados.

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Universidade do Minho, 31 de Março de 2017

Daniela Miranda Batista

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Abstract

The production and use of silver nanoparticles (AgNPs) has significantly grown over the last decade, increasing the risk that a fraction of NPs is transported to freshwater ecosystems. In these ecosystems, AgNPs and ionic silver released from dissolution of NPs can have toxic effects on aquatic species and compromise important ecosystem processes, such as plant litter decomposition. The research on the impacts of AgNPs on plant litter decomposition is scarce, rarely conducted under environmentally relevant conditions and not taking into account the physical and chemical properties of AgNPs that affect their toxicity.

The general goal of this study was to assess the impacts of AgNPs under environmentally-realist concentrations, considering species interactions and the physical and chemical properties of AgNPs at increasing temperatures focusing on freshwater detrital food webs.

Firstly, we assessed the impacts of AgNPs with different sizes and surface coating (100 nm PVP (polyvinylpyrrolidone)-dispersant, 50-60 nm and 35 nm uncoated) on freshwater decomposers of leaf litter by exposing leaf associated microbial assemblages to increasing concentrations of AgNPs and $AgNO_3$. We further conducted a feeding preference experiment with a common invertebrate shredder, *Limnephilus* sp., which was allowed to feed on microbially-colonized leaves previously exposed to AgNPs and $AgNO_3$. Leaf decomposition and microbial activity and diversity were inhibited when exposed to increased concentrations of 100 nm AgNPs, while microbial activity was stimulated by exposure to 35 nm AgNPs. Invertebrate shredders preferred leaves exposed to 35 nm AgNPs and avoided leaves exposed to $AgNO_3$. The 100 nm AgNP coated with PVP-dispersant were more stable than the uncoated AgNPs, indicating more aggregation and probably leading to lower toxicity. Our results highlighted the importance of considering the physical and chemical properties of NPs when assessing their toxicity to litter decomposers in freshwaters.

Secondly, we evaluated the effects of AgNPs at increased temperature on the activity and diversity of microbial decomposers of plant litter in streams and on the feeding behavior of invertebrate shredders. Litter-associated microbial communities were exposed in microcosms to increased concentrations of AgNPs/$AgNO_3$ and kept for 21 days at 10ºC, 16ºC and at 23ºC. Contaminated leaves were subsequently used to fed the invertebrate shredders *Limnephilus* sp. under the same temperature range, and after 5 days, animals were released from the stressor (AgNPs or $AgNO_3$) and allowed to feed on non-contaminated leaves. The increase in temperature stimulated leaf decomposition by microbial decomposers and shredders, and the activity of leaf degrading enzymes, while low temperature increased fungal biomass and diversity. Increased AgNP and $AgNO_3$ concentrations reduced reproduction and diversity of fungi. The negative effects on microbial activity were more pronounced at 10ºC and 23ºC, suggesting that changes in temperature might promote a severe threat of AgNPs to aquatic organisms.

Exposure of invertebrate shredders to leaves contaminated by AgNP induced oxidative stress in the animals. The activity of catalase (CAT) and superoxide dismutase (SOD) in the animals were associated with the total Ag accumulated, while glutationa transferase (GST) activity was strongly associated with treatments exposed to 23ºC. Results highlight the importance of considering different environmental scenarios when examining NP toxicity to freshwater biota.

Microbial communities can develop adaptive mechanisms toward tolerance against metals, so tolerance acquisition by these communities can be used as specific indicator of nano and ionic metal pollution in freshwater ecosystems. We conducted a pollution-induced community tolerance (PICT) approach to test if microbial decomposers had the ability to evolve tolerance to AgNPs. After 21 days of exposure to low concentrations of AgNPs and $AgNO_3$, microbial communities were able to acquire tolerance as measured via short-term bioassays using several microbial endpoints. Indeed, communities pre-exposed to AgNPs and $AgNO_3$ showed higher tolerance as revealed by the lower effects on fungal sporulation and bacterial biomass production. The results showed that approaches at the community level, such as PICT, using microbial decomposers might provide a better understanding of the mechanisms of toxicity triggered by NPs, helping to assess potential impacts of AgNPs on freshwater ecosystems.

Moreover, we assessed the impacts of environmentally-realistic concentrations of AgNPs and Ag^+ across a food web. Specifically, we assessed the importance of direct (via water) and indirect (via diet) exposure routes towards a simplified detrital food web, comprising leaf litter, microbes, a shredder species and collector species using: (i) water contaminated with AgNPs or $AgNO_3$), and (ii) leaves contaminated with AgNPs and $AgNO_3$. Microbial decomposition was lower by direct exposure to AgNPs and Ag^+, whereas leaf consumption by *Gammarus pulex* only decreased when leaves were contaminated with the lowest concentration of AgNPs. There were no effects on fine particulate organic matter produced by the shredder in both exposure routes. Changes in the activity of a key antioxidant enzyme, CAT, indicated AgNPs and Ag^+ caused oxidative stress in both invertebrate species, mainly in response to direct exposure. Overall results demonstrated that ecological effects on different functional groups of stream invertebrates vary with exposure route of AgNPs. Thus, the route by which stream biota are exposed to AgNPs will influence the impacts of AgNPs on ecosystem processes.

Impactos de nanopartículas de prata em cadeias tróficas detritívoras em ecossistemas de água doce num cenário de aquecimento global

Resumo

A produção e o uso de nanopartículas de prata (AgNPs) tem aumentado exponencialmente nos últimos anos, aumentando o risco de que uma porção destes nanomateriais possa ser transportada para os ecossistemas de água doce. As AgNPs e a prata iónica (Ag^+), que pode ser libertada destas NPs, podem ter efeitos tóxicos nas espécies aquáticas e nos processos por elas conduzidos, como é o caso da decomposição dos detritos vegetais. A investigação dos impactos das AgNPs na decomposição dos detritos vegetais em rios é ainda escassa, e raramente teve em conta condições ambientalmente realísticas a que as NPs estão sujeitas e, as características fisico-químicas das AgNPs.

O objectivo geral deste trabalho foi avaliar os impactos das AgNPs sob concentrações ambientalmente realísticas, considerando a interação de espécies e as propriedades fisico-químicas das AgNPs perante o aumento da temperatura, mais especificamente na cadeia trófica alimentar nos ecossistemas de água doce.

Primeiramente, foi avaliado o impacto das AgNPs com diferentes tamanhos e revestimentos (100 nm PVP-dispersante, 50-60 nm e 35 nm sem revestimento) nos decompositores dos detritos vegetais, nomeadamente microorganismos e invertebrados trituradores. Para tal os decompositores microbianos associados aos detritos vegetais foram expostos a concentrações crescentes de AgNPs e $AgNO_3$. A decomposição da folhada e a actividade e diversidade microbiana foram inibidos quando expostos a AgNPs de 100nm, enquanto que a exposição a AgNP de 35 nm estimulou a actividade microbiana. Os trituradores *Limnephilus* sp. mostraram preferência por folhas previamente expostas a AgNPs de 35 nm e evitaram folhas previamente expostas a $AgNO_3$. As AgNPs de 100 nm revestidas com PVP foram mais estáveis do que as não revestidas. Os resultados demonstram ainda a importância de considerar as características fisico-químicas das NPs quando se avalia a sua toxicidade para os decompositores em sistemas de água doce.

Seguidamente, foi testado o efeito do aumento de temperature na toxicidade das AgNPs para os decompositores microbianos e para os invertebrados trituradores. Durante 21 dias, as comunidades microbianas associadas à folhada foram expostas em microcosmos a concentrações crescentes de AgNPs e $AgNO_3$ e mantidas a 10ºC, 16ºC e 23ºC. As folhas contaminadas com AgNPs e $AgNO_3$ foram fornecidas como fonte de alimento a trituradores durante 5 dias nas mesmas temperaturas testadas. Após este período, os animais foram libertados do stress e alimentados com folhas limpas. O aumento da temperatura estimulou a decomposição pela comunidade microbiana e pelos trituradores, e a actividade de enzimas que degradam as folhas. As baixas temperaturas levaram ao aumento da biomassa e da diversidade de fungos. A exposição a AgNPs e $AgNO_3$ reduziu a

reprodução e a diversidade de fungos, sendo os efeitos mais pronunciados a 10ºC e a 23ºC. Os resultados sugerem que as mudanças de temperatura podem acentuar os efeitos das AgNPs para os organismos aquáticos. A análise da actividade enzimática nos trituradores demonstraram que a contaminação alimentar por AgNPs pode induzir stress oxidativo. A actividade da catalase (CAT) e da superoxide dismutase (SOD) estava associada com a acumulação de Ag nos animais, enquanto que a glutationa-transferase (GST) estava associada aos tratamentos expostos a 23ºC. Os resultados indicaram que a temperatura e a acumulação de Ag podem alterar a toxicidade das AgNPs, independentemente da via de exposição.

As comunidades microbianas podem desenvolver mecanismos que lhes conferem tolerância a contaminantes, podendo esta aquisição de tolerância ser indicadora da presença de contaminantes nos ecossistemas de água doce. O conceito de comunidade tolerante induzida por poluição (PICT) foi usado para testar se os decompositores microbianos conseguiam desenvolver tolerância a AgNPs. As comunidades microbianas foram expostas a concentrações baixas de AgNPs e $AgNO_3$ e após 21 dias a aquisição de tolerância foi analisada usando parâmetros microbianos. As comunidades expostas a AgNPs e $AgNO_3$ mostraram aumento da tolerância, como foi revelado pelos efeitos menores na reprodução de fungos e na produção de biomassa de bactérias. Assim, a avaliação dos efeitos das AgNPs ao nível das comunidades pode ajudar a perceber os mecanismos de toxicidade desencadeados pelas AgNPs.

Por último, foi avaliado o impacto de concentrações ambientalmente realistas de AgNPs e Ag^+ nas interações tróficas entre organismos envolvidos da decomposição de detritos vegetais em rios. Especificamente, foi estudada a importância da exposição directa (através da água) e indirecta (através de alimento) das AgNPs. Para isso, expusemos decompositores microbianos, e espécies de invertebrados pertencentes a diferentes níveis tróficos, trituradores e colectores a: (i) água contaminada com AgNPs e $AgNO_3$, e (ii) a folhas contaminadas com AgNP e $AgNO_3$. A decomposição microbiana foi reduzida através da exposição directa às AgNPs, enquanto que o consumo de folhas pelo triturador foi reduzido quando as folhas estavam contaminadas com a concentração mais baixa de AgNPs. Não houve efeitos na produção de matéria orgânica particulada fina pelos invertebrados em nenhuma das via de exposição. As alterações na actividade enzimática, especificamente da CAT, demonstrou que as AgNPs e a Ag^+ induziam stress oxidativo em ambas as espécies de invertebrados, especialmente através do alimento contaminado. Os resultados demonstraram que os efeitos ecológicos nos diferentes grupos funcionais variaram com a via de exposição às AgNPs em concentrações ambientalmente realísticas.

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Chapter 1
General introduction

1.1.Leaf litter decomposition in freshwater ecosystems

Decomposition of plant litter is a central ecosystem process in a wide range of terrestrial and benthic aquatic systems, including streams, littoral zones of lakes, and coastal marine environments (Cadisch and Giller, 1997; Gessner et al., 1997; Webster et al., 1999).

In low-order forested streams, primary production is very limit due to the low temperatures, low nutrient levels and low solar irradiation caused by density of riparian cover (Benfield, 1996; Mulholland et al., 2001). In these systems, e the major source of organic matter and energy is allochthonous and it is mainly composed of plant detritus (Cummins et al., 1974, 1989). This includes leaves and twigs that enter the streams and become available for aquatic biota or are transported downstream (Larrañaga et al., 2003; Elosegi, 2005).

When plant detritus enters the stream, it will be submitted to leaching, microbial conditioning, and biotic and abiotic fragmentation (Webster and Benfield, 1986; Gessner et al., 1999). Although these stages tend to occur sequentially in time, leaf decomposition is a complex process and some of these stages can occur simultaneously (Gessner et al., 1999; Allan and Castillo, 2007). In the leaching phase, soluble substances, such as phenolics, carbohydrates and amino acids, are lost between 24 hours and 7 days after leaf immersion, with mass loss reaching up to 25-30 % of initial mass, depending on the stream water variables, such as water temperature, turbulence and plant species (Gessner et al., 1999; Casas and Gessner, 1999). Conditioning corresponds to the colonization and growth of microorganisms on leaf litter that enhance leaf palatability to invertebrate shredders (Suberkropp, 1998; Graça, 2001). At this stage, leaves lose 10–30% of their initial mass and become softer while their polymers (cellulose, hemicelluloses, pectin) are attacked by fungal enzymes (Suberkropp and Klug, 1980; Bärlocher, 1985; Chamier, 1985). The fragmentation can be physical and biotic and results from the abrasion and shear stress exerted by the flowing water and from the feeding and digestive activities of microbes and invertebrate shredders that contribute to the release of fine particulate organic matter (FPOM) (Cummins, 1974; Allan and Castillo, 2007). At the end, the original plant material, constituted by coarse particulate organic matter (CPOM) is transformed into several products, including biomass of shredders, fungi and bacteria, dissolved organic matter (DOM), FPOM, inorganic nutrients and carbon dioxide (Gessner et al., 1999).

The organisms that drive these processes are microbial decomposers, mainly bacteria and fungi, and invertebrate detritivores, namely shredders and collectors (Bärlocher, 1992; Suberkropp, 1998; Gessner et al., 1999; Graça, 2001; Hieber and Gessner, 2002; Pascoal et al., 2005a). Among microbial decomposers, fungi, particularly aquatic hyphomycetes,

are known to play an important role in microbial decomposition of plant litter in streams, whereas bacteria are thought to increase their importance only after plant material has been partially broken down (Weyers and Suberkropp, 1996; Baldy et al., 2002; Pascoal and Cássio, 2004).

Aquatic hyphomycetes, also named Ingoldian fungi, are one of the most prominent groups of freshwater fungi (Barlöcher, 1992) and have the greatest ecological effects on freshwater ecosystems (Gessner et al., 2007). The morphological and physiological adaptations of these fungi are the reasons of their success as colonizers and decomposers of plant litter in streams: high conidial production and germination rates, the conidial shapes (tetraradiate or sigmoid) and the mucilage produced by their conidial arms allow an efficient dispersion and attachment to new substrata (Read et al., 1992). In addition, aquatic hyphomycetes remain active at low temperatures (Bärlocher and Kendrick, 1974), allowing adaptation to winter temperatures in streams of temperate climates (Suberkropp, 1998). Physiological adaptation includes the ability of producing a variety of extracellular enzymes able to degrade the structural constituents of vascular plants such as cellulose, hemicelluloses, pectin and, to a lesser degree, lignin, and consequently of transforming leaf material into a more suitable food source for stream detritivores (Bärlocher, 1992). Therefore, aquatic hyphomycetes are important mediators in the energy and nutrient transfer to higher trophic levels (Suberkropp, 1998).

Bacteria are also capable of producing enzymes that degrade the polysaccharides of plant litter (Burns, 1982), but its contribution to plant-litter decomposition is lower than that of fungi, when assessed either from biomass production or productivity (Gessner and Chauvet, 1994; Baldy et al., 2002; Hieber and Gessner, 2002; Pascoal and Cássio, 2004; Pascoal et al., 2005a). The relatively low contribution of bacteria to leaf decomposition may be related to its reduced or absent invasive ability, confining bacteria to the plant litter surface (Porter et al., 1989). Despite the differences in the contribution to leaf decomposition, fungi and bacteria can have interactions along plant litter decomposition. Bacteria are reported to grow better in the presence of fungi than alone (Romaní et al., 2006). However, aquatic fungi can produce antibiotics that limit bacterial growth (Gulis and Stephanovich, 1999) and, bacteria can produce fungicides or chitinolytic enzymes that suppress fungal growth (Romaní et al., 2006). In polluted rivers with depressed fungal activity, bacteria can increase its relative contribution to leaf-litter decomposition (Pascoal and Cássio, 2004).

Invertebrate detritivores also play a very important role in leaf decomposition: they fragment leaves and produce a large quantity of fecal pellets, producing large amounts of FPOM, which may constitute an important food source for other organisms called "deposit feeders" and "filter feeders" (Graça, 2001). Shredders have preference to feed on leaves conditioned by microbes, particularly fungi (Suberkropp, 1992; Graça, 1992) and they also grow faster, survive better and have a larger reproductive output when leaves are fully colonized by fungi (Graça et al., 1993). The reason is clear: microbial colonization after a period of conditioning leads to an increase in leaf nitrogen content (because of microbial biomass) and to leaf maceration, making the leaves more palatable for the animals (Suberkropp, 1992; Graça et al., 1993; Graça, 1993).

Furthermore, consumption of leaves by shredders appears to be affected by the type of fungal species colonizing the leaves (Bärlocher and Kendrick, 1973; Arsuffi and Suberkropp, 1985; Graça et al., 1993; Lecerf et al., 2005) and even by the leaf species (Canhoto and Graça, 2008). Several studies demonstrated that higher shredder abundance and biomass have been associated with faster leaf breakdown (Robinson and Gessner, 2000; Graça, 2001) and exclusion of shredders in streams, by application of an insecticide, led to a decrease in leaf breakdown rates (Wallace et al., 1996).

Several studies demonstrate that decomposers activity and leaf decomposition in streams depend on leaf litter quality (Sampaio et al., 2001) and several environmental factors such as temperature (Suberkropp and Weyers, 1996; Fernandes et al., 2009b), concentration of dissolved nutrients (Gulis and Suberkropp, 2003; Duarte et al., 2009; Fernandes et al., 2009a), and pH (Dangles et al., 2004). The impacts of stressors on leaf litter decomposition in streams have been a focus of interest over the last decades: a strong reduction in leaf decomposition as a result of the presence of metals (Niyogi et al., 2001; Duarte et al., 2008) or nanoparticles (Pradhan et al., 2011) has been observed. Being a complex process involving interactions between microorganisms and invertebrates, factors interfering with the activities of these organisms are likely to affect the functional process of plant litter decomposition compromising aquatic food webs and ecosystem functioning.

1.2. Silver nanoparticles in freshwater ecosystems.

In the last few decades, the progress of nanotechnology allowed nanomaterials to be a part of a wide range of manufactured commercial and domestic products, being used with potential benefits as sensors for environmental monitoring, nano-drug delivery,

biorobotics, nanoarrays and nano-scale implants in medicine (Roco, 2003; Rickerby and Morrison, 2007).

Nanomaterials (NMs) are materials that present at least one of their dimensions 100 nm or less and have unique properties and behavior different from bulk material usually due to its high surface-to-volume ratio resulting in high surface reactivity. Studies estimate that more than 1,300 products that are on the market today contain NMs (Bondarenko et al., 2013) NMs can be divided into five compound classes: carbonaceous nanomaterials; metal oxides; semiconductor materials, including quantum dots; zerovalent metals such as iron, silver, and gold; and nanopolymers, such as dendrimers. Within metal-containing nanomaterials, metal oxides, such as silver nanoparticles (AgNPs) are among the most commonly used nanoparticles (NPs) in consumer and industrial applications (www.wilsoncenter.org) due to their unique physicochemical characteristics, such as optical, electrical and biological properties, strong broad-spectrum of antimicrobial and anti-inflammatory activity, as well as relatively low manufacturing cost.

Since centuries ago, it is known that silver (Ag) has excellent antimicrobial activities mainly due to silver ion release (Ag^{+}) (Fan and Bard, 2002; Russell and Hugo, 1994). Therefore, silver nitrate ($AgNO_3$) and silver sulphadiazine (SSD) (Atiyeh et al., 2007) are widely used in medicinal products for preventing or treating infections/diseases (Fung and Bowen, 1996; Zhang et al., 2015) caused by viruses, fungi, and bacteria (Marx and Barillo, 2014). As a result, AgNPs is used mainly for antimicrobial purposes (Behra et al., 2013; Cascio et al., 2015; Slane et al., 2015), being present in materials for food packaging, water disinfectants, air filters, coating on medical devices, electronic appliances, odor-resistant textile fabrics, and cosmetic products such as deodorants and toothpaste; baby products; vacuum cleaners; and washing machines (www.wilsoncenter.org).

In 2012, the production of AgNPs in Europe was estimated to be in the range of 5.5 tons per year, and the worldwide consumption of silver nanoparticles was predicted to be 55 tons per year (Piccinno et al., 2012) or even higher (i.e., 360 tons per year to 450 tons per year) (Lazareva and Keller, 2014). More recently, the Nanoparticle Database has concluded that 68 AgNP products currently exist among the 645 commercialized single-element nanoparticles worldwide (Nanowerk LLC, 2015).

The rapidly expanding use of AgNPs in industrial applications and consumer products, arouse the concern of their release into aquatic ecosystems, either as their nano form or ionic Ag released from NPs (Blaser et al., 2008; Lapresta-Fernández et al., 2012). AgNPs are released from AgNPs coated socks during washing process (Benn and Westerhoff,

2008), where accumulative silver mass of up to 2 mg from 100 to 500 mg of socks was detected after washing for four times. Blaser et al. (2008) reported that up to 15% of total Ag in the form of silver ions (Ag^{+}) or AgNPs could be released from biocidal plastics and textile into water and Gottschalk et al. (2009) warns that AgNPs will most likely be sequestered in biosolids in properly operating wastewater treatment plants. Additionally, AgNPs can also be released by spillages, by intentional release for environmental remediation applications, or as end-of-life waste (Keller et al., 2013).

There is no certainty about the AgNP concentrations that can occur in the environment, but a number of risk assessment efforts have been made to model and calculate predicted environmental concentrations (PECs) of nanoparticles, reporting values for AgNPs ranging from ng L^{-1} to µg L^{-1} in the surface waters (Gottschalk et al., 2009, 2013; Blaser et al., 2008; Mueller and Nowack, 2008). For example, PEC values for AgNPs are around 0.72 ng L^{-1} in surface water in Switzerland (Gottschalk et al., 2010), and 3.3 µg L^{-1} for influent and 0.08 µg L^{-1} for effluent in wastewater treatment plants in England (Johnson et al., 2011).

Ag is considered one of the most toxic metals in freshwater environments (Ratte, 1999; Wood et al., 1999), and therefore it is regulated by various environmental regulations (Das et al., 2014; Thurman et al., 1989). For instance, Ag is listed in the EEC 1976 Dangerous Substance Directive List II (76/464/EEC), and the maximum Ag level for drinking water is set to 10 µg L^{-1} (80/778/CEE). To protect aquatic life and human health, the U.S. Environmental Protection Agency (U.S. EPA) set the acute water quality criteria for Ag in freshwaters to be 3.2 mg L^{-1} (U.S. EPA, 2009).

On the other hand, originally NPs were not included in the scope of Regulation (EC) No. 1907/2006 (REACH) (European Parliament, 2006), but there are ongoing discussions within the REACH competent authorities and its subgroup on nanomaterials on how REACH applies to nanomaterials (CA/59/2008 rev. 1; Brussels, 16 December 2008; European Commission, 2008). In 2008, AgNPs were included in the list of the 14 representative manufactured nanomaterials for testing (ENV/JM/MONO (2008)13/REV) defined by Working Party on Manufactured Nanomaterials (WPMN) stablished by The Organization for Economic Co-operation and Development (OECD) Council. Moreover, the adaptation of OECD test guidelines (TG) for aquatic and terrestrial ecotoxicity was proposed for testing NMs at an OECD WPMN expert meeting (OECD, 2014). More recently, significant progress on providing experimental data to support proposals for the adaptation of several OECD TGs was achieved by the project MARINA

(http://www.marinafp7.eu/), funded within the 7th European Framework Program (FP7) (Hund-Rinke et al., 2016).

AgNPs have unique properties and despite the wide number of toxicological studies on these NPs, there is still a lack of scientific consensus and uncertain about their mechanisms of toxicity and especially on which concentrations they can cause impacts on aquatic organisms.

1.2.1. Physical and chemical properties of AgNPs

AgNPs have exclusive properties (optical, electrical and magnetic properties) that make them of particular interest for several applications such as antimicrobial applications, biosensor materials, composite fibers, cryogenic superconducting materials, cosmetic products and electronic components (Caro et al., 2010).

The unique properties of AgNPs are mainly due to their small size and large surface-to-volume ratio, offering them high surface energy and more possible reactive sites, qualifying AgNPs as one of the most promising materials in catalysis. AgNPs also show surface plasmon resonance (SPR) under irradiation of light, inducing SPR peaks in the UV-vis wavelength range, that in which turn are influenced by the size, shape and dispersion of the nanoparticles (Tolaymat et al., 2010). These unique properties make possible the use of AgNPs in sensing and imaging applications, including the detection of DNA (Harper et al., 2012).

The physical and chemical properties, including size, shape, surface area, solubility and type of coatings are particularly important for determining their biological interactions and impacts. The shape of AgNPs can dramatically affect their properties and it is generally assumed that smaller particles have larger surface area and, therefore, greater toxic potential (Scown et al., 2010; Angel et al., 2013; Silva et al., 2014). Also, the presence or absence of coating on AgNPs influences their toxicity, with some coatings being more toxic than others (Angel et al., 2013; Zhao and Wang, 2012a).

Toxicological research demonstrated that the behavior of AgNPs is influenced not only by inherent factors, such as NP size, shape, surface area, surface chard, crystal structure, coating, and solubility/dissolution, but also by environmental factors including temperature, pH, ionic strength, salinity and organic matter (Levard et al., 2012; Navarro et al., 2008a; Zhang et al., 2016). For example, charge-stabilized AgNPs (e.g., citrate coating) are more susceptible to external conditions than sterically stabilized AgNPs (polyvinylpyrrolidone: PVP or polyethylene glycol: PEG) (Tejamaya et al., 2012; Cupi et

al., 2016). Ionic strength is also an important factor for the stability of AgNPs, of which high ionic strength tends to weaken the electrostatic repulsion between particles and reduce the electric double layer on the surface of AgNPs, resulting in colloidal aggregation (Jin et al., 2010). The pH influences the surface potential of NPs (Cupi et al., 2016), and the presence of various functional groups of natural organic matter (NOM) facilitates surface binding of AgNPs, resulting in more stable AgNP suspensions (Huynh and Chen, 2011).

The uptake and toxicity of AgNPs, depend on environmental factors. Consequences of AgNP exposure for aquatic ecosystems need to include the complex ecological and physicochemical context of natural environments (Holden et al., 2016; Hund-Rinke et al., 2016). This realism is lacking in laboratory-scale studies where single species are often exposed to AgNPs under controlled and simplified environmental conditions (Ali et al., 2014; Walters et al., 2014; Zhao et al., 2011).

1.2.2. Behavior of AgNPs in water

AgNPs can enter the aquatic environment (Blaser et al., 2008; Fabrega et al., 2011; Siripattanakul-Ratpukdi and Fürhacker, 2014) by several ways: a) Ag leached out from AgNP consumer products; (b) suspended AgNPs in air and then depositing in the water; (c) runoff scouring AgNP polluted soils or land and sewage sludge.

Several studies have focused on how the AgNP properties can be affected by the aquatic environment and the main transformations that have been reported are: aggregation, oxidation and dissolution, sulfidation and chlorination (Yu et al., 2013; Behra et al., 2013; Zhang et al., 2016).

Particle aggregation

Aggregation process is affected by numerous environmental factors, such as pH, ionic strength and composition, NOM, temperature, and nanoparticle concentration, all interacting and affecting aggregation or stabilization of Ag NPs (Navarro et al., 2008a; Zhang et al., 2016). Manipulation can control the aggregation of particles, for instance by using coating materials, such as citrate or even synthetic and natural polymers such as PVP (Hirai et al., 1979).

The pH significantly influences the stability of AgNP suspensions (Badawy et al., 2010; Keller et al., 2010; Musee et al., 2014; Cupi et al., 2016) and the toxicity, which is also related to coating. For example, Badawy et al. (2010) observed that the same pH could minimally impact the aggregation of PVP coated AgNP, but similar changes in pH

significantly influenced the aggregation of branched polyethyleneimine (BPEI) coated AgNPs.

Increases in ionic strength can significantly increase the aggregation rate of AgNPs (Badawy et al., 2010; Levard et al., 2012), being more significant for particles with smaller size (Harmon et al., 2014). Aggregation is also induced by sunlight (Cheng et al., 2011) or even by the presence of dissolved oxygen (DO) (Zhang et al., 2011). Moreover, certain organic substances can induce a charge to AgNP surface, increasing AgNPs stability. For instance, humic acids (HA) from a river at 10 mg L^{-1} caused disaggregation of AgNPs (Fabrega et al., 2009a).

Particle size to which organisms are exposed is determined by aggregation, which in turn can either reduce bioavailability to organisms or might favor exposure via ingestion (Battin et al., 2009; Fabrega et al., 2009b). It is generally assumed that aggregation can reduce AgNP toxicity (Zhao and Wang, 2012b) but aggregated AgNPs are less mobile and deposited in the sediments, where can be taken up by filter-feeders and sediment-dwelling organisms, and can potentially result in biomagnification in the food chain (Frabega et al., 2011).

Oxidation and dissolution

Dissolution of AgNPs to Ag ions is a result of surface oxidation and is one of the most important steps of AgNP transformation in aquatic environments. With this transformation possible toxic products, such as Ag chloride (AgCl), Ag_2S, Ag_2O, and Ag(I) complexes can be formed. The Ag dissolution depend on the chemical and surface properties of the particle as well as on its size (Misra et al., 2012), and it is also affected by several environmental factors, such as dissolved oxygen, pH, temperature and salinity (Grady et al., 2011).

In oxygenated water, AgNPs is quickly oxidized, and a layer of Ag_2O can be formed on its surface, generating a “core shell” structure and preventing the dissolution of AgNPs (Chen et al., 2006; Li et al., 2010; Yin et al., 2002). Liu and Hurt (2010) found that removing dissolved oxygen from AgNP suspensions, the release of Ag^+ was completely inhibited, and the dissolution of Ag^+ was greatly enhanced by decreasing pH in air-saturated water, showing that both protons and dissolved oxygen played key roles in controlling Ag^+ release.

Smaller AgNPs tend to release Ag ions faster than larger ones, enhancing the particle toxicity (Scown et al., 2010; Shang et al., 2014). The nature of the coating is also important

in AgNP dissolution; however, appears to be less significant than the size effect. For instance, dissolution of PVP coated and tannic acid coated is more significant than that of citrate coated AgNPs (Dobias and Bernier-Latmani, 2013).

AgNPs are expected to be more stable in freshwaters at low ionic strength, such as in crystalline rock areas, and to be increasingly destabilized in freshwater with higher ionic strength (in carbonate rock areas), as in estuarine waters and in seawater. It is expected that dissolution will be most efficient at low pH (less than 7) and in the presence of strong binding ligands for Ag^+. Bioavailability of Ag^+ is decreased by the presence of strong binding ligands, including inorganic sulfide and organic thiol ligands (Behra et al., 2013).

Sulfidation and chlorination

Sulfidation can occur promoting changes in surface properties and morphology, as seen by Levard et al. (2011) which monitored the sulfidation process of PVP-coated AgNPs in Na_2S solutions. The immersion of an antimicrobial sock containing AgNPs into a hypochlorite/detergent solution, more than 50% AgNPs in socks were converted to AgCl in hypochlorite/detergent solution (Impellitteri et al., 2009), showing that chlorination process is a possible transformation of AgNPs.

Additionally, regeneration is a recent transformation discovered by some authors (Glover et al., 2011; Yin et al., 2012), which implied that AgNPs could be generated from Ag^+ spontaneously in nature (Yin et al., 2012).

1.2.3. Characterization of AgNP and its importance

A wide number of toxicological studies about AgNPs have been published, but the majority has been performed without proper AgNP characterization, making it difficult to take conclusions about their toxicity (Navarro et al., 2008a; Handy et al., 2012; Bandyopadhyay et al., 2012; Walters et al., 2014). Moreover, it is very difficult to do comparisons between studies and generalizations that allow further use of data without understanding the characteristics of AgNPs.

Most studies on AgNPs rely on information of commercial products with limited characterization on the stock suspensions, sometimes with unidentified capping agents, with little knowledge of potential impurities or stability. The characterization of AgNPs should include several properties: size distribution, aggregation, surface properties (charge, chemistry, specific area, etc), morphology (shape, crystal structure and purity), dissolution rate and solubility (Fabrega et al., 2011). Additionally, these properties should ideally be

quantified in real exposure situations, taking into account the experimental conditions (medium) and exposure periods of the experiment, in order to establish the causes of toxicity and the mode of action of NPs (Domingos et al., 2009; Holden et al., 2016). Behra and Krug (2008) underline the importance of a correct choice of NPs to use in biological experiments, and the tests needed to characterize them before, during and after the experiments. A proper characterization of the NP should be performed using the latest techniques, and at least following the recommendations in the OECD WPMN document (OECD, 2012).

AgNP average size and size distribution can be determined by several techniques, such as transmission electron microscopy (TEM) (Mavrocordatos et al., 2005; Domingos et al., 2009; Leppard, 2008), scanning electron microscopy (SEM), atomic force microscopy (AFM) (Balnois et al., 2007), confocal optical microscopy, and X-ray diffraction (XRD) and dynamic light scattering (DLS) (Domingos et al., 2009; Schurtenberger and Newman, 1993).

Sample preparation for TEM analysis is quite laborious and the particle size can be determined only in regions that are translucent to an electron beam. TEM can produce bright-field or dark-field images that can provide precise particle size information at the nanoscale. On the other hand, it can occur an overestimation of particle sizes, which result from a bias of the operator to particles that are easier to see. Sample preparation for SEM is simpler than for TEM; however, problems with interpretation can occur when analyzing dispersed nanocrystallites (Wilkinson, 2013).

DLS technique is one of the most frequently used methods to characterize size. DLS measures fluctuation of scattering dependency of particles undergoing Brownian motion to determine size distribution profiles. DLS is fast (minutes) and can be used in a range oscillating from 1nm to 10μm; however, large particles cause large deviation problems, a single large particle or agglomerate will have a disproportionate effect on the signal. While algorithms have been developed to correct for polydispersity, they invariably make numerous assumptions and are rarely appropriate for more than two distinct size classes (Filella et al., 1997). DLS measurements may need to be complemented by determinations from other techniques, such as nanoparticle tracking analysis (NTA) (Domingos et al. 2009; Gallego-Urrea et al., 2009). NTA is a real-time visualization technique based on a laser-illuminated microscope coupled to a charge-coupled device camera, which tracks individual particles based on their Brownian motion by use of the Stokes-Einstein equation. This technique is able to measure two independent parameters simultaneously, such as

particle-scattering intensity and particle diameter. Recent studies have used NTA technique as a validation of the results obtained with DLS (Tlili et al., 2016; Gil-Allué et al., 2015), since NTA appears to provide a more accurate and thorough size distribution of polydispersed samples compared to DLS (Filipe et al., 2010).

Characterization of AgNP surface charge is also important to predict their agglomeration and binding to different substances. One of the techniques used to characterize the dispersability of NPs is through the zeta potential. Using this technique, it is possible to measure the change in the surface charge of NPs at different pH values and ionic strengths, their aggregation in solution, and their binding to environmental surfaces (Keller et al., 2010). Zeta potential is determined with the electrophoretic mobility through photon correlation spectroscopy or high-performance capillary electrophoresis, which measures differences in electricity in electrolyte solutions (Fan et al., 2006; Kuo and Lin, 2006). Changes in zeta potential indicate the instability of the NPs, changes in particle concentration (Tantra et al., 2011), or the nature of the material that is under characterization (Peltonen and Hirvonen, 2008).

In ecotoxicological studies, determining whether the organisms are being exposed to dissolved Ag, NPs or even NPs aggregates is still a major challenge. It is crucial to discriminate the Ag forms (dissolved, NP or "bulk" particles) for defining a NPs mode of action and understanding hazard issues. Changes in the physiochemistry of the medium can have large effects on the Ag forms. Furthermore, quantification of the different Ag forms is also needed. Analytical methods such as induced couple inductively coupled plasma mass spectrometry (ICP-MS) can be very accurate in quantification of Ag but does not distinguish its form (Tlili et al., 2016). Combinations of TEM and EDX are another approach to resolve this problem, but are time consuming and costly (Pradhan et al., 2011). Physical techniques, such as filtration or ultrafiltration, are used to separate dissolved particulate or colloidal NPs. Although these techniques are used to rapidly and easily distinguish between dissolved and NPs, more promising techniques are being tested for NP samples: cross-flow filtration (Guo and Santschi, 2007), centrifugal filtration devices (e.g., Centricon), or forced dialysis (e.g., Slide-A-Lyzer). Ultracentrifugation is also used to separate NPs from the dissolved phase (Gil-Allué et al., 2015; Tlili et al., 2016). In that case, the particle separation will depend upon the particle density (with respect to the experimental medium), the centrifugal force, and the time that particles are centrifuged (Stokes' law).

Even though, these techniques only measure the free ion present in the samples and this quantification alone may often be insufficient to quantify exposure and too concluded about the risk. Therefore, estimation of metal speciation is commonly used by several authors (Gil-Allué et al., 2015; Tlili et al., 2016). Programs for metal speciation modeling such as MINEQL+, MINTEQA2, and WHAM are widely used by aquatic chemists and ecotoxicologists, but they require knowledge on chemical reactions, good selection of stability constants, and, of course, measurements of key parameters describing water chemistry, such as pH, inorganic and organic carbon concentrations, and major ions (Na^+, Ca^{2+}, Mg^{2+}, K^+, SO_4^{2-}, Cl^-).

A major challenge is the characterization of AgNPs in the environment, because detection depends upon the nature and size of the particles, the physicochemical properties of the suspension, and the basic principles of different analytical techniques (Handy et al., 2012). Although there are many analytical methods available to characterize, only a few can be used to determine AgNPs in environmentally relevant concentrations. Usually the techniques have high limits of detection (Marinella et al., 2011), not sufficient for natural waters where concentrations are generally in the sub-nanomolar range (Sigg et al., 2006). AgNPs at environmentally relevant conditions should be measured with very sensitive techniques and should be able to minimize sample disturbance to ensure that the laboratory conditions represent the unaltered environmental state. A review paper from Bandyopadhyay et al. (2012) describes several techniques and relative problems to a proper characterization of environmental NPs. No single technique allows a complete unambiguous characterization of complex samples and therefore it is endorsed the use of multiple instruments or techniques.

In conclusion, several aspects should be taken into consideration for a proper NP characterization, such as: (1) characterize the AgNPs in stock solution and medium at least at the beginning and end of the exposure period; (2) choose multiple techniques, combined or not, to enhance reliability of the results; and (3) take into account the possible Ag forms (dissolved, NP or “bulk” particles) and their interactions with the study media. Despite the evolution of the techniques for AgNP characterization, these aspects are overestimated for many researchers and there are still several obstacles to overcome them.

1.2.4. Effects of AgNPs on aquatic biota and plant litter decomposition in streams

The growing production of AgNPs raise concerns about the potential impacts of AgNPs released in nano or ionic form into the freshwater ecosystems. In recent years, several review papers have addressed the environmental effects of AgNPs (Dorobantu et al. 2015; Fabrega et al., 2011; Navarro et al., 2008a; Zhang et al., 2016).

Silver is one of the most toxic metals to both freshwater and marine organisms, highly persistent in the environment and with a great capacity to accumulate in water, sediments and organisms (Ratte, 1999; Fabrega et al., 2011; Luoma, 2008). Toxicity of ionic Ag is well documented causing significant mortality to fish and aquatic invertebrates at μg L^{-1} and even ng L^{-1} levels (Ratte, 1999). The mechanisms of ionic Ag toxicity to aquatic organisms is characterized by the high affinity of Ag^{+} for thiol groups, which causes the ion to interact strongly with membranes and enzymes, affecting essential physiological processes such as photosynthesis, respiration and other enzymatically catalyzed processes. On the other hand, the mechanisms of AgNP toxicity are poorly understood. The toxicity of AgNPs can be attributed to the nano properties (such as the small size), or mediated by dissolved Ag^{+} ions and other dissolved silver species derived from AgNP, such as $AgCl^{-}$ (aq) and AgOH (aq) (Marambio-Jones and Hoek, 2010; Levard et al., 2012; Yu et al., 2013). Studies demonstrated that the effects of AgNPs are less pronounced than those of the respective Ag ions (Pradhan et al., 2011; Navarro et al., 2015). For example, Navarro et al. (2008b) showed that AgNP alone had minimal toxicity to the freshwater algae *Chlamydomonas reinhardtii* and served mostly as a source of Ag^{+}. However, several authors reported that leached Ag ions are insufficient in explaining the toxicity of NPs due to low solubility of some metal ions (Griffitt et al., 2008; Wang et al., 2012; Tlili et al., 2016). For instance, Griffitt et al. (2008) concluded that Ag had an enhanced toxicity to freshwater cladocerans (*Ceriodaphnia dubia*) if present as AgNP.

AgNP can exert toxic effects also on a wide range of organisms, such as plants (Gubbins et al., 2011), bacteria (Kim et al., 2007), algae (Navarro et al., 2015), fungi (Kim et al., 2008; Tlili et al., 2016), vertebrates (Asharani et al., 2008; Kim et al., 2013), and invertebrates (Andreï et al., 2016; Roh et al., 2009; Zhao and Wang, 2011). In microorganisms, AgNP exposure can significantly damage cellular structures (cell membranes and cell walls), cellular components (nucleic acids, lipids, and proteins), and/or important cellular functions (metabolic activities) (Hackenberg et al., 2011; Siripattanakul-

Ratpukdi and Fürhacker, 2014; Yu et al., 2013; Zhao and Wang, 2012). In particular, the cell membrane/wall might be the main biological target of AgNPs (Zhang et al., 2016).

The presence of AgNPs influenced the bacterial community within marine biofilms by reducing the development and succession of normal bacterial biofilm within 4 days of growth (Fabrega et al., 2009b). Recently, AgNPs toxicity was demonstrated in periphyton, with the suppression of extracellular enzyme activity (leucine aminopeptidase) even in the presence of a chelating ligand showing a specific particle effect of AgNPs (Gil-Allué et al., 2015). Moreover, Oukarroum et al. (2012) observed ROS formation and lipid peroxidation (LPO) in the freshwater microalgae *Chlorella vulgaris* and marine microalgae *Dunaliella tertiolecta* when assessing the toxic effects of AgNPs.

AgNPs cause sublethal phenotypic abnormalities in embryos of aquatic vertebrates (Asharani et al., 2008; Kim et al., 2013; Laban et al., 2010) and have been shown to be cytotoxic and genotoxic to different cell types, with effects including membrane damage, oxidative stress, metabolism disruption, DNA damage and apoptosis (Choi et al., 2010; Wise et al., 2010; Baker et al., 2014). AgNPs can also accumulate in the gills and liver tissues affecting the ability of fish to cope with low oxygen levels and inducing oxidative stress (Bilberg et al., 2010; Scown et al., 2010).

Many other aquatic organisms have also been examined and information on the ecotoxicity of AgNPs in aquatic organisms has been recently summarized (Lapresta-Fernandez et al., 2012; Dorobantu et al., 2015).

Microbial decomposers

Although adverse effects of metal ions (zinc, cadmium, silver) on litter decomposition and associated microbial communities have been clearly established (Duarte et al., 2008; Moreirinha et al., 2011; Sridhar et al., 2005), the toxicity of AgNPs to microbial decomposers and litter decomposition has received little attention (Pradhan et al., 2011; Tlili et al., 2016). Microbial decomposers have a critical role in organic matter and nutrient cycling in aquatic ecosystems, where AgNPs can potentially alter ecosystem productivity and biochemistry by affecting the aquatic fungi. Indeed, the exposure to AgNP led to a reduction in fungal sporulation and leaf decomposition (Pradhan et al., 2011). A recent study by Tlili et al. (2016) clearly demonstrated the high toxicity of AgNP to microbial decomposers of plant litter by disrupting bacterial growth and clearly affecting fungal growth, with both endpoints being more sensitive to AgNPs than to Ag^{+}. In contrast, fungal

reproduction was stimulated by at certain AgNP concentrations, emphasizing the importance of using a wide range of AgNP concentrations in the experiments.

Shifts in the composition of leaf-associated fungal assemblages based on fungal spore morphology and DNA fingerprints were already observed with AgNPs exposure. *Articulospora tetracladia* was stimulated, while species like *Flagellospora* sp. was inhibited by exposure to nano or ionic Ag, being replaced by *Heliscus lugdunensis* (Pradhan et al., 2011). Some aquatic fungi can be found in streams affected by metal pollution (Pascoal et al., 2005a; Sridhar et al., 2001). Indeed, some fungal species were found to be resistant to high concentrations of metals in their ionic or nano form and additionally develop mechanisms to cope with the oxidative stress induced by metal nanoparticles (CuO-NPs: Pradhan et al., 2015a).

If microbial decomposers or their competitive or trophic interactions are affected by AgNPs, then larger consequences could ensue for the structure of stream food webs and ecosystem processes, and the overall flow of energy in ecosystems. Effects on microbial decomposers are expected to translate to higher trophic levels. Uptake or incorporation of AgNPs into microbial biomass suggests the potential for trophic transfer of these contaminants as observed in studies with CuO-NPs (Pradhan et al., 2012, 2015b).

Freshwater invertebrates

Silver is recognized as a toxic metal for small aquatic invertebrates (Ratte, 1999´; Bianchini et al., 2003; Funck et al., 2013). Recently, Funck et al. (2013) demonstrated toxicity of $AgNO_3$ to the freshwater Crustacea *Gammarus fossarum* even for environmentally realistic concentrations (0.5 mg L^{-1}), with significant effects on their physiology (decrease of haemolymph osmolality) and behavior (decrease in locomotor and ventilatory activities).

Research about the AgNPs impacts on freshwater invertebrates has received little importance, especially on invertebrate detritivores where the tests have been focused on acute effects and mainly performed with daphnids (Zhao and Wang, 2011, 2012a; Völker et al., 2013) and gammarids (Andreï et al., 2016; Mehennaoui et al., 2016). Concurrently, some studies observed detrimental effects of AgNPs on growth and reproduction of daphnids (Zhao and Wang, 2011; Mackevica et al., 2015) and *Chironomus riparius* (Nair et al., 2011; Park et al., 2015), highlighting the need of more quantitative assessments of the chronic and sublethal effects of AgNP. Recently, a study observed a functional impact of AgNPs through the decrease of FPOM production by a gammarid at very low

concentrations (0.5 µg L^{-1}), suggesting that AgNPs may induce a change in gammarid energy provision (Andreï et al., 2016).

Some of the research on invertebrate species has shown how the type of capping of the AgNPs, ionic strength and concentration of organic carbon in the media are crucial for predicting and understanding AgNP toxicity. For instance, differences in mortality of daphnids was observed among three different surface coatings of AgNPs and the bioaccumulation of AgNPs in daphnids depended on the NP characteristics, such as particle size and surface coating (Zhao and Wang, 2012b). Furthermore, the aquatic toxicity of AgNP to different organisms (algae, cladoceran and freshwater fish larva) was mitigated by the presence of organic matter (Wang et al., 2015).

Considerable number of studies have reported toxicity of ionic and nano Ag to freshwater invertebrates mainly through waterborne exposure (Blinova et al., 2013; Ali et al., 2014) but less is known on the effects through dietary exposure (Croteau et al., 2011; Zhao and Wang, 2011; Mouneyrac et al., 2014).

Zhao and Wang (2011) conducted an experiment where daphnids were exposed to waterborne and dietborne AgNPs, observing that the waterborne AgNP had the most significant inhibition on growth, whereas dietborne had mainly effects on reproduction. The chronic effects of AgNP were probably caused by the low food quality of algae associated with AgNP and the low depuration of ingested AgNPs.

In toxicity tests with other freshwater invertebrate, Croteau et al. (2011) investigated the bioaccumulation dynamics in the snail *Lymnaea stagnalis* following both aqueous and dietary exposure to AgNPs. In the diet, AgNPs interfere with the digestion, and snails ate less and inefficiently processed the ingested food, which adversely impacted their growth. Moreover, *L. stagnalis* efficiently accumulated Ag from water and diet sources.

Therefore, determining the bioavailability, uptake and accumulation of AgNPs in aquatic organisms is essential for the evaluation of their toxicity to aquatic life and transfer along the food chain. Invertebrates are able to intake NPs dispersed in the environment by different ways: via food ingestion or from contaminated preys, or direct from water filtration, inhalation, and surface contact.

Only a limited number of studies have reported so far on cellular internalization of AgNPs in aquatic organisms. AgNPs were detected in the gut epithelium of the deposit feeder *Nereis diversicolor* feeding on sediments contaminated by AgNPs (García-Alonso et al., 2011), and in daphnids more than 70% of AgNPs were accumulated through ingestion

(Zhao and Wang, 2011) emphasizing the significance of the transport of NPs along the aquatic food chain.

Additionally, new founds suggested changes in gene expression induced by AgNPs, especially in oxidative stress-related genes or DNA damage-related genes in *C. riparius* (Nair et al., 2013; Park et al., 2015). Poyton et al. (2012), using a transcriptomic approach in *Daphnia magna*, confirmed the specific gene expression response caused by PVP AgNPs and also that AgNPs cause different gene expression profiles compared with $AgNO_3$, suggesting different mechanisms of action.

1.3. Effects of climate change on freshwater ecosystems

Climate change (CC) is considered one of the major threats to biodiversity, ecosystem functioning and services they provide (Dudgeon et al., 2006; Vörösmarty et al., 2010). CC is represented by the occurrence of extreme weather events such as increases in extreme high temperatures, decreases in extreme low temperatures, and increases in drought periods followed by intense rainfalls (Easterling et al., 2000; Jentsch et al., 2007). Some regions such as North and South America, northern Europe, and northern and central Asia are projected to experience increased precipitation, while others, including southern Africa and Asia and the Mediterranean, are expected to experience substantial droughts (Noyes et al., 2009). CC can indirectly affect aquatic ecosystems via geomorphological alterations of lake and river systems, changes in nutrient and ionic loads (leading towards alterations of photosynthetic rates, eutrophication, acidification, salinization) as well as enhancing the impacts of prevalent diseases, chemical pollutants, biological invasions, and changes in predation and competition among species (IPCC, 2007).

The recent IPCC report (2013) anticipates an increase in mean global air temperature of 2.6–4.8ºC by the end of this century. Reports about the consequences of increasing temperature in several organisms point to changes in physiology, time of life cycle events, and distribution of individual species with shifts toward higher altitudes or latitudes (Parmesan and Yohe, 2003; Root et al., 2003). Moreover, it is probable that species will become extinct, either as a direct result of physiological stress or via alterations in competitive interactions with other species (Hughes, 2000).

There is a consensus in the scientific community that freshwater ecosystems would be particularly vulnerable to CC (Carpenter et al., 1992; Dudgeon et al., 2006; Ormerod et al., 2010), especially in streams where increase in average water temperature and changes in precipitation and stream discharge (Murdoch et al., 2000; Schiller et al., 2011) are possible

phenomena. Stream water temperature is expected to follow air trends, with an increase up to 0.6ºC– 0.9ºC for each degree in air temperature (Morrill et al., 2005; Webb and Nobilis, 2007).

In freshwater ecosystems, most organisms strongly depend on temperature (Parmesan and Yohe, 2003; Heino et al., 2009; Perkins et al., 2010), and it has been shown that changes in temperature can alter species phenology and body size distributions across food webs (Hogg and Williams, 1996; Friberg et al., 2009; Woodward et al., 2010). The consequences of these changes include shifts in species traits, trophic structure (Petchey et al., 1999) and, consequently, the magnitude of the biological contribution of these species to ecosystem processes (Domingos et al., 2015).

Leaf litter decomposition is a process expected to be particularly sensitive to changes in water temperature, as it depends strongly on temperature (Davidson and Janssens, 2006). Laboratory experiments demonstrated that higher water temperatures affect litter decomposition, directly by promoting leaching of soluble compounds (Chergui and Pattee, 1990), and by enhancing microbial activity (Carpenter and Adams, 1979) and indirectly, by stimulating fragmentation or enhancing leaf consumption by selected invertebrate shredders (González and Graça, 2003; Azevedo-Pereira et al., 2006; Ferreira et al., 2009).

Some research was done about the effect of temperature on the growth and sporulation of aquatic hyphomycetes. Laboratory experiments suggest that an increase in temperature stimulate the production of fungal assemblages on leaves (Suberkropp and Weyers, 1996) and growth and sporulation of some species of aquatic hyphomycetes (Koske and Duncan, 1974; Graça and Ferreira, 1995; Rajashekar and Kaveriappa, 2000; Dang et al., 2009; Ferreira and Chauvet, 2010), which in turn can accelerate litter decomposition rates (Batista et al., 2012; Fernandes et al., 2009b; Ferreira and Chauvet, 2010). Higher temperatures generally stimulate enzymatic activities (Canhoto et al., 2016) and fungal respiration rates (Ferreira and Chauvet, 2010; Ferreira et al., 2012; Gonçalves et al., 2013). Moreover, temperature increases potentiate litter mass loss stimulating the carbon mineralization. On the other hand, the exposure to temperatures close to 0ºC leads to a distinct reduction in biomass and conidium production of aquatic hyphomycetes (Bärlocher and Kendrick, 1974; Buttimore et al., 1984; Nikolcheva and Bärlocher, 2005). Fernandes et al. (2009b) conducted a microcosms experiment assessing the effects of extreme weather event (increasing temperature or freezing) on leaf-litter associated fungi and the results suggested that occasional freezing is likely to constrain diversity and the ecological functions of aquatic fungi, while warming appears to accelerate plant-litter decomposition in streams.

Temperature appears to be an important factor affecting the occurrence and distribution of aquatic fungi (Suberkropp, 1984). Some species are more common in temperate climates, and others are more common in the tropics (Bärlocher, 1992; Goh, 1997). For example, *Flagellospora penicillioides* and *Lunulospora curvula* do not grow below 5ºC, disappearing from streams during colder seasons (Webster et al., 1976; Suberkropp, 1984). Indeed, in microcosm experiments, changes in temperature often lead to shifts in the structure of stream-dwelling microbial communities (Dang et al., 2009; Fernandes et al., 2009b, 2012; Batista et al., 2012), with fungal species typical of warmer waters becoming dominant on decomposing plant litter under warming treatments (Fernandes et al., 2009, 2012), while freezing can cause extinction of typical summer species such as *Lunulospora curvula* (Fernandes et al., 2009b). Moreover, composition and activity of fungal communities adapted to cold environments were more affected by the temperature increase than the fungal communities adapted to warm environments (Martínez et al., 2014; Duarte et al., 2016). Dang et al. (2009) suggesting that effects caused by diel temperature oscillations can be significant even when changes in the community structure are relatively minor (i.e., only relative proportions of species in communities changed) and the dominance of particular species in a diverse community may alter the response of ecosystem process rates to warming under naturally oscillating temperature regimes.

Invertebrates are also highly responsive to CC, with their responses well documented in terrestrial, marine and freshwater systems (e.g., Hogg and Williams, 1996; Bale et al., 2002; Parmesan and Yohe, 2003; Zvereva and Kozlov, 2006; Prather et al., 2013). The direct effects of CC include changes in geographic distributions, population size, phenology, behavior, and genetic composition (Parmesan, 2006). CC can also affect indirectly the invertebrates: for example, changes in plant quality due to increased atmospheric CO_2 concentrations will alter food quality for the detritivores, which in turn will alter growth, survival, and/or feeding rates in detritivorous invertebrates in aquatic and terrestrial environments (Tuchman et al., 2002; Zvereva and Kozlov, 2006).

The increase in temperature is expected to lead to several effects on invertebrates, such as faster initial growth rates, shorter developmental time and smaller size at maturity (reviewed by Atkinson, 1995; Atkinson and Sibly, 1997). The effects of increased water temperature are predicted to be stronger for invertebrates inhabiting cold waters when compared with those inhabiting warmer waters (Braune et al., 2008), because in cold water environments biological activities are more temperature limited (Brown et al., 2004). In a field manipulative experiment, Ferreira et al. (2015) found that warming stimulated the

activity of macroinvertebrate individuals and stimulated decomposition of leaf litters in winter, while Domingos et al. (2015) found an inhibitory effect of raised temperature on macroinvertebrate activity with important consequences for leaf litter decomposition in spring. Authors defend that the increase in the stream water temperature affect the consumption by the invertebrate shredders which preferentially feed on leaves colonized by certain fungal species (Lecerf et al., 2005; Canhoto and Graça, 2008; Chung and Suberkropp, 2009; Gonçalves et al., 2014). Despite the benefit that invertebrates may have with the increased conidium production induced by raised temperature (Bärlocher and Brendelberger, 2004), changes in mycelium quantity and quality affect detritus palatability (Lecerf et al., 2005; Canhoto and Graça, 2008) and digestibility through accelerated fungal-mediated degradation (Bärlocher et al., 2008).

However, an opposite trend can also be found: Moghadam and Zimmer (2016) obtained no effects in the relative growth rates of *Gammarus pulex* exposed to an increase of 5ºC in temperature. Data from a global experiment in streams through a latitudinal temperature gradient found that warming will likely hasten microbial litter decomposition and produce an equivalent decline in detritivore-mediated decomposition rates, resulting in unchanged decomposition rates (Boyero et al., 2011).

Changes in consumption rates, lower invertebrate growth rates, higher mortality and changes in body composition under extreme weather events may result in a decrease of populations with impacts to the processes in which these organisms are involved (e.g., litter decomposition, nutrient cycling). In a large-scale outdoor pond, Jonsson et al. (2015) observed a reduction in the number of emergent Chironomidae, while Trichoptera and Ephemeroptera remained unchanged, with only an increase of 3ºC above ambient temperatures.

Moreover, CC has a great effect on the distribution and biological effects of chemical toxicants by altering physical, chemical, and biological drivers of partitioning between the atmosphere, water, soil/sediment, and biota, including: air-surface exchange, wet/dry deposition, and reaction rates (e.g., photolysis, biodegradation, oxidation in air; MacDonald et al., 2002). Temperature has the largest influence on the partitioning of chemical toxicants, by altering exposure pathways and increasing susceptibility of some populations, especially those already under stress (Gaston et al., 2003; AMAP, 2004; Breivik et al., 2004; Macdonald et al., 2005). Interactive effects between temperature and other stressors have attracted considerable attention, such as changing supplies of nutrients (Fernandes et

al., 2009a, 2012; Ferreira and Chauvet, 2010) or metal pollution (Boukadida et al., 2016; Batista et al., 2012; Ferreira et al., 2012).

So far, the impacts of AgNPs and changes in temperature have been predominantly examined independently and the interaction between these two stressors is poorly investigated so the information on their combined effects is lacking. Studies have shown that temperature tends to enhance the toxicity of metals on leaf decomposition (Batista et al., 2012), but so far only a few demonstrated the enhanced toxic effect of AgNPs by temperature on aquatic algae (Oukarroum et al., 2012). A study by Walters et al. (2016) reported for the first time the co-effects of AgNPs and temperature on crabs (*Potamanautes perlatus*): data indicated that AgNP-mediated toxicity to *P. perlatus* is modulated by elevated temperatures although this relationship was not linear. Moreover, the combined effects of temperature and toxicants are expected to yield complex results at the community level, but so far there are few studies on these interactions that also involve multiple species (Moe et al., 2013).

1.4. Microbial adaptation to contaminants: a pollution induced community tolerance (PICT) approach

Nowadays, ecotoxicology research is largely limited to laboratory studies with single-species in highly controlled conditions (Morones et al., 2005; Amin et al., 2009). However, studies based on organismal responses to stressors are clearly insufficient to predict the impact of stressors to the entire community and associated ecological processes (Batista et al., 2012). Therefore, studies at the community level are warranted if we are to elucidate the effects of stressors on ecosystem functioning.

Blanck et al. (1988) proposed the concept of pollution-induced community tolerance (PICT) as an ecotoxicological tool that provides a good approach for environmental status characterization, useful not only for assessing immediate impacts but also for taking into account the ecosystem contamination history at the community level (Dorigo et al., 2004; 2010).

Microbial communities have biological and ecological specificities that make them very interesting tools for environmental quality assessment (Tlili and Montuelle, 2011). Being composed of many species with different sensitivities to stressors (Clements and Rohr, 2009), when microbial communities are exposed to a stressor, some species may acclimate or develop adaptation, increasing the whole community tolerance to that or related stressors (Vinebrooke et al., 2004). This process underlies the concept of PICT: a chronic exposure

to toxicants lead to an increase in the community tolerance that might result from adaptation or acclimatization of populations or from shifts in community composition that results from the replacement of sensitive species by tolerant species (Blanck, 2002). In principle, the PICT concept can be applied to all types of ecosystems, aquatic or terrestrial (Hjorth et al., 2006; Niklinska et al., 2006), and to all microbial communities such as bacteria (Boivin et al., 2005) or photosynthetic microorganisms (Dahl and Blanck, 1996).

The application of the PICT concept involves two phases (Molander and Blanck, 1991): a selection phase is based on how communities adapt to stress conditions, in which a community is exposed to a toxicant, either in its natural habitat or in a laboratory experiment; and a detection phase where the community is exposed to the toxicant once again in a short-term assay (without any selection) in order to determine whether any change in tolerance has occurred.

In selection phase the exposure of communities to toxicants leads to the selection of the most tolerant species, and this tolerance acquisition depends not only on inter- and intra-specific diversity but also on the specific sensitivities of the strains or species that comprise the community. Changes in microbial communities are used to confirm structural differences and diversity evolution between communities affected or not by the toxicant. Taxonomic analysis of algal communities (Bérard and Benninghoff, 2001) has been used, but also non-taxonomic methods can provide interesting evidence for microbial diversity changes. Molecular biology tools are also used, such as polymerase chain reaction (PCR) on 16S rDNA (prokaryotes) and 18S (eukaryotes), followed by denaturing gradient gel electrophoresis (DGGE) (Dorigo et al., 2007; Duarte, Pascoal and Cassio, 2009) or even high-pressure liquid chromatography (HPLC), which allows the identification of photosynthetic pigments of major algal groups, allowing investigation of the structural changes of photosynthetic communities (Dorigo et al., 2007).

In the detection phase community tolerance is determined by the means of short-term toxicity tests based on physiological activities (for instance respiration and photosynthesis). Community tolerance is usually expressed as the EC_{50} value derived from such a short-term test and the comparison of the obtained EC_{50} value with that of an unexposed (reference) community clarifies whether or not the toxicant has affected the community (Blanck and Dahl, 1996).

To detect tolerance acquisition, functional parameters are considered because its relevance as an ecotoxicological endpoint depends first on the mode of action of the toxicant (and its bioavailability) and second on the mechanisms involved in the community tolerance.

Parameters measuring primary production are usually used to assess tolerance in aquatic photosynthetic organisms in both the phytoplankton and the phytobenthos (Dahl and Blanck, 1996; Bérard et al., 2003). Other studies have focused on heterotrophic bacteria by measuring bacterial DNA synthesis (Blanck et al., 2003) or microbial respiration (Tlili et al., 2010). Moreover, the induction of algal fluorescence by photosynthesis inhibitors, and recently the measure of antioxidant enzyme activities in several organisms (Niklinska et al., 2006; Fechner et al., 2012; Bonnineau et al., 2013) are also used.

The acquisition of PICT may vary with many factors, such as target community, intensity of toxicant exposure, the species succession stage, and the physicochemical characteristics of the studied site, because of the structural and functional complexity that characterizes biological communities. There are important steps to take into account in the PICT approach: for example, the type and the duration of exposure to a toxicant are crucial for the development of tolerance. Schmitt et al. (2005) demonstrated that two weeks exposure to Zn and Cd was not sufficient to observe PICT development in bacterial communities, unlike the six weeks exposure. PICT studies have been mainly conducted with small organisms with fast life cycles, mainly algae and bacteria, facilitating the analysis of short-term contamination effects (pulse contamination) (Tlili et al., 2008). Another important step is the choice of the reference community, which should be able to acquire tolerance after exposure to a toxicant (Blanck et al., 1988). Interferences from environmental factors should be account when leading to the PICT approach. The initial community had an original reference condition (e.g., nutrients, temperature, and light) that should be maintained for minimize the interference in the detection of PICT. Some studies have demonstrated the effects of nutrients (Ivorra et al., 2002; Brandt et al., 2009) and temperature (Boivin et al., 2005) on the community tolerance acquisition.

Despite the advantage of community tolerance, this can be an undesirable phenomenon. Co-tolerance can occur when communities that have been exposed to one toxicant, but not to another, become tolerant to both toxicants (Blanck et al., 1988). Even more, the adaptation to environmental stressors may increase community sensitivity to novel stressors, suggesting a potential cost associated with tolerance acquisition (Wilson, 1988; Clements and Rohr, 2009). The elimination of the most sensitive species from a community may induce an alteration of the ecosystems functions and an augmentation of the sensitivity of the community to an additional perturbation, reducing the ability of that community to respond to future disturbances (Paine et al., 1998). Kashian et al. (2007) showed that benthic communities subjected to long-term metal pollution were generally more tolerant

to metals but more sensitive to UV-B radiation than communities from a reference site, suggesting a potential fitness cost associated with increased metal tolerance (Wilson, 1988). For aquatic organisms, first reports dealing with tolerance were published for algae exposed to metals in the 1950s and for bacteria in the 1970s (quoted in Klerks and Weis, 1987). Moreover, bacteria and fungi are frequently tolerant to higher levels of metals (Abel and Bärlocher, 1984; Baldy et al., 2002; Guimarães-Soares et al., 2007; Azevedo et al., 2009). In case of aquatic hyphomycetes, despite they have been mainly documented in clean streams (Bärlocher, 1992), they have also been found in metal-polluted streams. These fungi have shown evidence of developing adaptive mechanisms toward tolerance/resistance against metals, such as higher production of tiol-containing proteins/peptides (Guimarães-Soares et al., 2007) and regulated cell death pathway (Azevedo et al., 2009), which helps them to survive in harsh environments. Pradhan et al. (2015a) demonstrated that CuO-NPs were less toxic for fungi isolated from metal polluted streams than from non-polluted streams. Unfortunately, the mechanisms underlying such differences have not yet been investigated, but previous studies observed a change in the structure of fungal communities in stream microcosms after exposure to metals and to CuO-NPs (Pradhan et al., 2011).

PICT approach has been used on several studies: terrestrial microbes (Díaz-Ravina and Bååth, 1996; Bérard et al., 2014); freshwater biofilms (Tlili et al., 2011; Bonet et al., 2014); and algal communities (Soldo and Behra, 2000; McClellan et al., 2008), but no studies were reported in microbial communities involved in plant litter decomposition.

Besides providing an ecotoxicological tool, PICT approach might be a useful tool for risk assessment (Blanck et al., 1988) since it takes into account the species interactions by working at the community level, giving more ecologically relevant information on pollutant effects in the environment (Blanck, 2002). Indeed, the PICT approach can help to reveal a causal relationship between a contaminant and its ecological effects (Rutgers and Breure, 1999). Finally, PICT has been used to demonstrate community recovery from environmental contamination, reflecting its relevance as an indicator in ecosystems restoration projects (Dorigo et al., 2010; Rotter et al., 2011).

1.5. Aim and outline of the thesis

The growing production and use of AgNPs over the last years raised the concern of their release into freshwater ecosystems, where AgNPs and ionic Ag released from NPs can have toxic effects on aquatic species and compromise important ecosystem processes. In this

study, we assessed the impacts of AgNPs on leaf-litter decomposition and the associated biota involved in this process, such as microbial decomposers (fungi and bacteria) and the invertebrate shredders. Since the toxicity of AgNPs depends on several factors, the behavior of AgNPs under different environmental scenarios, and the physical and chemical properties of AgNPs were investigated. Additionally, microbial tolerance, exposure routes, and trophic interactions were also taken into account to test AgNP toxicity.

Chapter 1 provides an overview on plant litter decomposition in streams and the associated biota. Particular attention is given to AgNPs impacts on freshwater ecosystems with a brief description on the physical and chemical properties of AgNPs and their behavior in aquatic systems. The impacts of AgNPs under the ongoing global warming on aquatic biota involved in plant litter decomposition were also addressed. Another issue addressed was to describe how microbial communities are able to evolve tolerance to contaminants, such as AgNPs.

In Chapter 2, a microcosm assays was carried out to assess the impacts of AgNPs with different size and surface coating (100 nm PVP-dispersant, 50-60 nm and 35 nm uncoated) on freshwater microbial decomposers by exposing leaf associated microbial assemblages to increasing concentrations of AgNPs and of $AgNO_3$. In addition, a feeding preference experiment with an invertebrate shredder was performed to check how the different properties of NPs affect the feeding behavior of invertebrate shredders that feed on leaf litter previously colonized by microbial decomposers.

The main goal of Chapter 3 was to assess the responses of microbial decomposers to AgNPs under different environmental scenarios. A microcosm experiment was conducted by exposing microbial decomposers of plant litter to increasing concentrations of AgNPs and increased temperature. The functional parameters examined were: leaf mass loss, fungal biomass, from ergosterol content in leaves, and fungal reproduction, by counting spores released from decomposing leaves. Also, the stress induced by AgNPs and $AgNO_3$ on litter-associated microbial communities was assessed by measuring the activity of extracellular enzymes involved in the decomposition of plant components such as cellulose or lignin. The interactive effects of AgNPs and temperature on the structure of aquatic hyphomycete assemblages were assessed based on the diversity of sporulating fungi and on DNA fingerprints from denaturing gradient gel electrophoresis.

In Chapter 4, we assessed the impacts of AgNPs and $AgNO_3$ at higher trophic levels of detrital food webs by measuring the feeding activity of invertebrate shredders and their oxidative stress responses after exposure to microbially-colonized leaves contaminated

with AgNPs and Ag^+ under increasing temperatures to test whether changes in temperatures modulates the AgNPs effects. A common species of invertebrate shredders was allowed to feed on leaves contaminated with AgNPs and $AgNO_3$, and incubated at different temperatures (10ºC, 16ºC and 23ºC). Moreover, the effects on these parameters were also determined after the animals were released from the stressor (AgNPs and $AgNO_3$) and allowed to feed on clean leaves.

In Chapter 5, the ability of microbial decomposers to evolve tolerance to AgNPs was determined by exposing these communities to low concentrations of AgNPs and ionic Ag. Tolerance acquisition was measured via short-term bioassays using several microbial endpoints: fungal sporulation, bacterial production, microbial respiration and the activity of leucine aminopeptidase.

In Chapter 6, the impacts of environmentally-realistic concentrations of AgNPs and Ag^+ on trophic interactions between aquatic invertebrates and consequent ecosystem effects were assessed. Moreover, the importance of different exposure routes (via water and via diet) was considered by exposing a simplified detrital food web, comprising leaf litter, microbes, a shredder species and collector species to AgNPs and $AgNO_3$. Besides leaf consumption and FPOM produced by invertebrates, we also assessed fungal biomass and activity on leaves. Finally, the stress induced by AgNPs and Ag^+ in both invertebrate species was assessed by measuring the activity of antioxidant and neuronal enzymes.

Moreover, along the chapters the potential bioaccumulation along the food-web was examined by quantifying AgNPs in the stream water, leaf litter and invertebrates.

The main conclusions are presented in Chapter 7, to provide a global perspective of the study and possible lines for future research.

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Chapter 2

How do physicochemical properties influence the toxicity of silver nanoparticles on freshwater decomposers of plant litter in streams?

Batista D, Pascoal C, Cássio F. 2017. How do physicochemical properties influence the toxicity of silver nanoparticles on freshwater decomposers of plant litter in streams? Ecotoxicology and Environmental Safety. 140:148–155

Abstract

AgNP physicochemical properties may affect AgNP toxicity, but their effects on plant litter decomposition and the species driving this key ecosystem process in freshwaters have been poorly investigated. We assessed the impacts of AgNPs with different size and surface coating (100 nm PVP (polyvinylpyrrolidone)-dispersant, 50-60 nm and 35 nm uncoated) on freshwater decomposers of leaf litter by exposing leaf associated microbial assemblages to increasing concentrations of AgNPs (up to 200 mg L^{-1}) and of $AgNO_3$ (up to 25 mg L^{-1}). We further conducted a feeding preference experiment with a common invertebrate shredder, *Limnephilus* sp., which was allowed to feed on microbially-colonized leaves previously exposed to AgNPs and $AgNO_3$. Leaf decomposition and microbial activity and diversity were inhibited when exposed to increased concentrations of 100 nm AgNPs ($\geq$ 25 mg L^{-1}), while microbial activity was stimulated by exposure to 35 nm AgNPs ($\geq$ 100 mg L^{-1}). Invertebrate shredders preferred leaves exposed to 35 nm AgNPs (25 mg L^{-1}) and avoided leaves exposed to $AgNO_3$ ($\geq$ 2 mg L^{-1}). Results from the characterization of AgNPs by dynamic light scattering revealed that AgNps with PVP-dispersant were more stable than the uncoated AgNPs. Our results highlight the importance of considering the physicochemical properties of NPs when assessing their toxicity to litter decomposers in freshwaters.

2.1.Introduction

Silver nanoparticles (AgNPs) have been increasingly used over the last decade (PEN, 2013) mainly due to their strong antimicrobial activities and other unique physiochemical properties (catalytic activity, Jiang et al., 2011; specific electronic properties, Dubey et al., 2009). AgNPs are incorporated in textiles, detergents, personal health care products, and also have several medical applications. In Europe, the production of AgNPs in 2012 was 5.5 tons (Piccinno et al., 2012), and the Nanoparticle Database reported that 68 AgNP products exist among the 645 commercialized single-element NPs worldwide (Nanowerk LLC, 2015). The growing production of AgNPs arouse the concern of their release into freshwaters, where AgNPs and ionic Ag released from these NPs might be toxic to aquatic species (Moore, 2006; Navarro et al., 2008a; Fabrega et al., 2011) compromising the ecological processes they drive (e.g., organic matter decomposition, Pradhan et al., 2011).

The decomposition of allochthonous organic matter, such as riparian plant litter, is a key process in freshwater ecosystems. This organic matter is degraded by fungi and bacteria and, subsequently, incorporated into food webs (Graça, 2001). Fungi, particularly aquatic hyphomycetes, play a key role in plant litter decomposition in streams (Baldy et al., 2002; Pascoal and Cássio, 2004) because they produce a variety of extracellular enzymes that degrade complex polysaccharides of plant cell walls, including cellulose, hemicellulose and lignin, making leaf material a more appropriate source of carbon and energy for invertebrate shredders (Suberkropp, 1998), which in turn transfer energy to higher trophic levels (Graça and Canhoto, 2006).

The few studies on the impacts of AgNPs on plant litter decomposition showed that leaf decomposition, microbial biomass and fungal reproduction are inhibited by exposure to nano and ionic Ag (Pradhan et al., 2011). These studies suggested that the increased release of nano metals to the environment might affect aquatic microbial communities with impacts on organic matter decomposition in streams. On the other hand, considerable number of studies have reported lethal toxicity of ionic and nano Ag to aquatic invertebrates mainly through waterborne exposure (Zhao and Wang, 2012; Blinova et al., 2013; Silva et al., 2014; Ali et al 2014), but less is known on sublethal effects such as their feeding activity (Croteau et al., 2011; Zhao and Wang 2011; Mouneyrac et al., 2014).

Another concern is the behavior of AgNPs in water that is known to be influenced either by physical and chemical water parameters, such as temperature, ionic strength and pH (Walters et al., 2013; Siripattanakul-Ratpukdi and Fürhacker, 2014) or by physical and chemical characteristics of NPs, such as particle hydrophobicity, concentration and size

(Navarro et al., 2008b; Sharma et. al, 2014; Zhang et. al, 2016). The toxic effects of AgNPs have shown high inter-study variability, even contradictory effects, which may be partly explained by differences in particle size and specific surface area, NP shape and aggregation, composition of test media among others (Siripattanakul-Ratpukdi and Fürhacker 2014). There is evidence that AgNPs with smaller size tend to have higher toxicity (Scown et al., 2010; Angel et al., 2013; Silva et al., 2014). However, AgNPs with large diameter (10 nm) shortened the lifespan of *Caenorhabditis elegans*, causing lethal damage, whereas the small size (2 nm) of AgNP only affected nematode fertility (Contreras et al. 2014). Also, the presence of coating greatly influences the fate, stability and toxicity of AgNPs (Zhao and Wang, 2012; Sharma et al., 2014). In freshwater algae and daphnids the citrate-coated AgNPs were more toxic than the PVP (polyvinylpyrrolidone) -coated AgNPs despite undergoing much greater aggregation (Angel et al., 2013), indicating that the type of coating affects toxicity while aggregation does not.

The aim of this study was to assess the impacts of particle size and coating of AgNPs on freshwater microbial decomposers of plant litter. To that end, microbial communities associated with leaf litter were exposed for 28 days to 4 concentrations of AgNPs with 3 different sizes (100 nm PVP (polyvinylpyrrolidone)-dispersant, 50-60 nm and 35 nm uncoated). The toxicity of $AgNO_3$ was compared to that of Ag ions released from AgNPs to determine if the observed toxicity was due to Ag dissolution from AgNPs or was particle specific. In addition to clarify how particle size and concentration of AgNPs could affect the feeding behavior of organisms that depend on the activity of microbial decomposers, a feeding preference experiment with an invertebrate shredder was performed.

We expected that i) the coated AgNPs would show higher physical and chemical stability than the non-coated AgNPs, so they would be less reactive and toxic, ii) toxicity would increase with decreasing AgNP size and increasing concentrations of AgNPs and $AgNO_3$, and iii) invertebrate shredders would avoid to feed on leaves previously exposed to AgNP or $AgNO_3$. Information on the comparative toxicity across different sizes, coating and concentrations of AgNPs will help us to better evaluate the impacts of AgNPs on freshwater ecosystems.

2.2.Material and methods

2.2.1. Experimental design

Leaves of *Quercus robur* L. (oak), collected in September 2011, were air dried and kept at room temperature. The leaves were leached in deionized water for 2 days and cut into 12

mm diameter disks. Sets of 60 leaf disks were placed into fine-mesh bags (0.5 mm pore size) and immersed in a low-order stream located in NW Portugal (Algeriz Stream, 41º 350'N 8º220'W), to allow microbial colonization.

During leaf immersion, conductivity (34 µS cm^{-1}), pH (6.4) and dissolved oxygen concentration (98% saturation) were measured in situ using a Multiline F/set 3 no. 400327 (WTW). Stream water samples were collected and transported (4ºC) to the laboratory, and analysed (HACH DR/2000 spectrophotometer, Loveland, CO) to determine the concentrations of nitrate (0.02 mg $N\text{-}NO^{-}_{3}$ $L\text{-}^{1}$), nitrite (0.0035 mg $N\text{-}NO^{-}_{2}$ L^{-1}), ammonia (<0.01 mg $N\text{-}NH_3$ L^{-1}) and phosphate (0.01 mg PO_4^{3-}-P L^{-1}).

After 10 days of stream immersion, leaf bags were returned to the laboratory for microcosm experiments. Leaf disks were rinsed with deionized water and placed in 150 mL Erlenmeyer flasks with 80 mL of sterile stream water. The information on the stream water used in the microcosms experiment can be found in Pradhan et al. (2015b).

To test for AgNPs and $AgNO_3$ toxicity, the microcosms were supplemented with increasing concentrations of AgNPs of different sizes (35 nm, 50-60 nm and 100 nm) or ionic metal as follows: 25, 50, 100 and 200 mg L^{-1} of AgNPs; and 2, 5, 15 and 25 mg L^{-1} of $AgNO_3$ ($AgNO_3$, >99%, Sigma-Aldrich, St. Louis, MO). All treatments were done in triplicates and additional microcosms not supplemented with AgNPs were used as control. The microcosms were kept under shaking (120 rpm) at 16ºC and solutions were renewed every 7 days until the end of the experiment. After 28 days, leaf disks were freeze-dried to determine leaf mass loss, fungal biomass and diversity as described below.

2.2.2. Preparation and characterization of AgNPs

AgNPs tested were: AgNP spherical particles with a particle size < 100 nm, containing PVP (polyvinylpyrrolidone) as dispersant, specific surface area of 5 m^2/g, and purity of 99.5% based on trace metal analysis (CAT no. 7440-22-4, from Sigma-Aldrich, St. Louis, MO); AgNP spherical particles with a particle size 35 nm, specific surface area of 30-50 m^2/g and purity of 99.5% based on trace metal analysis (NM-0023-HP-0010, IoLiTec Ionic Liquids Technologies, GmbH, Germany); and AgNP spherical particles with a particle size 50-60 nm, specific surface area of ~12 m^2/g, and purity of 99.9% based on trace metal analysis (NM-0038-HP-0010, IoLiTec Ionic Liquids Technologies, GmbH, Germany).

The stock suspensions of each AgNPs size (35 nm, 50-60 nm and < 100 nm) was prepared according to Pradhan et al. (2012). The hydrodynamic diameters of the AgNPs in the stock suspensions, in the medium freshly prepared and after 28 days of exposure were measured

by dynamic light scattering (DLS) using a Zetasizer (Nano ZS, Malvern Instruments Ltd., Worcestershire, UK). The zeta-potential of the AgNP suspensions was measured using the Zetasizer.

2.2.3. Metal analysis

Total Ag concentration in the medium and leaf disks was determined (CACTI, University of Vigo, Spain), after acid digestion, by inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer Optima 4300 DV, U.S.). Dissolved Ag from the AgNP suspensions was determined after ultrafiltration (30 min at 3220 g, Megafuge 1.0R, Thermo Scientific Inc., Waltham, MA) using Ultracel 3k Centrifugal Filter Devices (Amicon Millipore) with a molecular cutoff of 3 kDa (pore size <2 nm) and the Ag concentrations in the filtrate were measured for total Ag.

2.2.4. Leaf decomposition

To determine leaf mass loss, freeze-dried (Christ alpha 2–4; B. Braun, Melsungen, Germany) leaf disks from each replicate before and after stream colonization, and after microcosm exposure were weighed to the nearest 0.001 mg.

2.2.5. Activity of plant litter degrading enzymes

The extracellular enzymes analyzed were β-glucosidase involved in the last step of cellulose degradation; and phenol oxidase involved in the break down of plant fibers such as lignin. The activity of the extracellular enzymes was measured at 2 intermediate concentrations of AgNPs (25 and 100 mg L^{-1}) and $AgNO_3$ (2 and 25 mg L^{-1}).

The enzyme β-glucosidase (EC 3.2.1.21) was analyzed using fluorescent (MUF, methylumbelliferone)-linked artificial substrate (MUF-β-D-glucopyranoside, Sigma). Colonized leaf disks (2 disks per microcosm) were incubated at saturating concentrations of the substrate (0.3 mM) for 1 h in the dark at 16ºC immediately after retrieval from the microcosms. Blanks and standards of MUF (0–100 µM) were also incubated. At the end of the incubation, glycine buffer (pH 10.4) was added (1:1 vol/vol), and the fluorescence was measured at 455 nm upon excitation at 365 nm (Spectra Max Plus 384, Molecular Devices, Sunny Vale, CA, USA).

Phenol oxidase activity (EC 1.10.3.2 and 1.14.18.1) was measured using L-3,4-dihydroxyphenylalanine (L-DOPA, Sigma) according to Sinsabaugh et al. (1994). Incubations (2h) were performed with 5 mM L-DOPA concentration with acetate buffer

(pH 5.0). Samples (2 leaf disks) were incubated at 16 ºC under shaking in dark. Blanks and standards of L-DOPA (0–10 mM) were also incubated. Absorbance was measured at 460 nm with a fluorimeter (Fluoroskan Ascent FL; Labsystem, Helsinki, Finland). Values are expressed as nanomoles of MUF and L-DOPA per gram of dry mass of leaves.

2.2.6. Fungal biomass and diversity

Fungal biomass was estimated based on quantification of ergosterol on leaves according to Gessner (2005). Briefly, lipids were extracted from 7 leaf disks of each replicate by heating (80 °C for 30 min) in 8 g L^{-1} of KOH/methanol, purified by solid-phase extraction and the retained ergosterol eluted in isopropanol. Ergosterol in the extracts was further quantified by high performance liquid chromatography (UltiMate 3000 LC Systems, Thermo Scientific, CA, USA) with a UV detection at 282 nm. The extraction efficiency was monitored by using a standard series of ergosterol (Fluka) in isopropanol.

Fungal diversity was estimated by counting the fungal spores released during exposure of leaf disks to AgNPs and $AgNO_3$. The fungal conidia in suspensions were fixed with formaldehyde (2% final concentration) and Triton X-100 was added to avoid spore adherence to the flasks until counted. Appropriate aliquots were filtered (5 µm pore size, Millipore, Billerica, MA) and stained with 0.05% cotton blue in lactic acid. The fungal spores were then counted and identified under a light microscope (400x, Leica Biomed, Heerbrugg, Switzerland).

2.2.7. Feeding preference by invertebrate shredders

Feeding experiments were carried out with early-stage larvae of the shredder *Limnephilus* sp. (Trichoptera: Limnephilidae), which is a common invertebrate genus in Iberian streams. Animals with similar size, were collected from the upper reach of the Cávado River during autumn, acclimated to the laboratory (1 week) and starved for 24 h before the experiment. Detailed information on the Cávado River can be found in Pascoal et al. (2001).

After starvation the animals were exposed to sterile stream water and microbially-colonized leaf disks contaminated or not with AgNPs and $AgNO_3$ as follows: AgNPs (25, 50, 100 and 200 mg L^{-1}) with different sizes (35, 50-60 and 100 nm) and to $AgNO_3$ (2, 5, 15 and 25 mg L^{-1}). Leaves were pre-exposed to the treatments at 16 ºC for 28 days.

The feeding preferences of the animals were assessed not only by preference of AgNP/$AgNO_3$ concentrations but also by the AgNP size. To assess the preference by AgNP/$AgNO_3$ concentrations the animals had available in the same container leaves

contaminated by different concentrations of the same AgNP size or leaves contaminated by different $AgNO_3$ concentrations. To assess the preference by AgNP size, the animals had available in the same container leaves contaminated with different AgNP sizes but the same concentration.

For each replicate, 1 animal (14.5 X 1.8 mm length) and 10 leaf disks (2 leaf disks from each concentration/size treatment) were placed in plastic containers (15 cm x 4.5 cm) with 300 mL of sterile stream water, and the leaf disks of each concentration/size were marked with colored pins. All microcosms were aerated with constant air flow and incubated at 16 °C, under a 12 h light: 12 h dark photoperiod. The experiment was run for 5 days. Leaf material remaining after the feeding experiment was frozen and lyophilized to a constant weight. Animals were also frozen, dried (60 °C, 3 days) and weighed to the nearest 0.01 mg. Leaf consumption was measured as the difference between the initial and the final leaf dry mass.

2.2.8. Data Analysis

Two-way ANOVAs were used to determine the effects of exposure concentrations and sizes of AgNP, and one-way ANOVA was used to determine the effects of $AgNO_3$ concentrations on leaf mass loss, fungal biomass and reproduction, activity of extracellular enzymes, and leaf consumption by invertebrates (Zar, 2010). Significant differences between control and treatments were analysed by Bonferroni post-hoc tests (Zar, 2010). No data transformation was done since the data were homoscedastic and had a normal distribution. Graph Pad Prism 5 program (GraphPad software Inc., San Diego, CA) was used for all statistical tests described above.

2.3.Results

2.3.1. Nanoparticle characterization

The average particle diameter of nanoparticles with initial size of 100, 50-60 nm and 35 nm sizes were 158 ± 4, 240 ± 5 and 243 ± 34 nm, respectively, for the stock suspensions with a concentration of 1000 mg L^{-1} (Table 2.1). The average size was lower in microcosms at the beginning of the experiment than in the stock suspensions. Nanoparticle size tended to increase with the exposure time in microcosms with 100 nm AgNP, and to decrease in microcosms with 50-60 nm and 35 nm AgNP sizes. The greater AgNP size was observed after 28 days at the highest concentration and corresponded to 253 ± 49 nm, for AgNPs with initial sizes of 100. AgNPs in stock suspension of 100, 50-60 and 35 nm had a zeta-

potential of -31 ± 1, -25 ± 1 and -11 ± 1, respectively (Table 2.1). The zeta-potential was stable along the experiment for all AgNP sizes, except for 200 mg L^{-1} of AgNPs, in which the zeta-potential decreased after 28 days of exposure. The AgNPs seemed to be more stable in stock suspension than in the medium after 28 days of exposure, except for the 35 nm AgNP where an opposite trend was observed as indicated by the polydispersion index (PdI, Table 2.1).

Table 2.1 - AgNP particle diameter measured by dynamic light scattering (DLS) on coated (100 nm PVP) and uncoated (35 nm and 50 nm) AgNP stock suspensions (1000 mg L^{-1}) and on the medium, freshly prepared and after 28 days of exposure. The polydispersion index (PdI) and the zeta-potential was measured by Zetasizer. Mean ± SD, n = 3.

Sample			DLS		Zeta potential (mV)
NanoSize (nm)	Concentration (mg L^{-1})	Time (days)	Diameter (nm)	PdI	
35	1000	-	243 ± 34	0.91 ± 0.12	-11 ± 1
	25	0	62 ± 16	1.00 ± 0.0	-29 ± 1
		28	66 ± 31	0.77 ± 0.15	-27 ± 7
	50	0	98 ± 15	1.00 ± 0.0	-31 ± 1
		28	105 ± 37	0.56 ± 0.15	-32 ± 4
	100	0	98 ± 15	1.00 ± 0.0	-31 ± 1
		28	133 ± 37	0.51 ± 0.07	-26 ± 1
	200	0	175 ± 27	0.94 ± 0.11	-32 ± 1
		28	138 ± 35	0.58 ± 0.07	-13 ± 3
50-60	1000	-	240 ± 5	0.37 ± 0.03	-25 ± 1
	25	0	167 ± 19	0.27 ± 0.03	-30 ± 2
		28	83 ± 13	0.71 ± 0.11	-33 ± 3
	50	0	177 ± 7	0.29 ± 0.01	-29 ± 1
		28	114 ± 47	0.57 ± 0.07	-32 ± 3
	100	0	213 ± 11	0.36 ± 0.04	-29 ± 1
		28	139 ± 41	0.59 ± 0.12	-31 ± 1
	200	0	202 ± 4	0.33 ± 0.02	-30 ± 1
		28	128 ± 30	0.52 ± 0.09	-9 ± 2
100 PVP	1000	-	158 ± 4	0.36 ± 0.02	-31 ± 1
	25	0	130 ± 2	0.32 ± 0.02	-34 ± 1
		28	130 ± 2	0.32 ± 0.02	-34 ± 1
	50	0	121 ± 3	0.29 ± 0.01	-32 ± 1
		28	217 ± 30	0.50 ± 0.10	-32 ± 2
	100	0	124 ± 2	0.29 ± 0.02	-30 ± 1
		28	226 ± 18	0.46 ± 0.04	-30 ± 2
	200	0	125 ± 5	0.27 ± 0.02	-30 ± 1
		28	253 ± 49	0.44 ± 0.08	-13 ± 2

At the end of the experiment, concentrations of Ag found in the leaves and in the medium exposed to AgNPs and $AgNO_3$ were particularly high in treatments with 50-60 nm AgNPs at 200 mg L^{-1} (8.25 ± 0.14 mg g^{-1} and 70.63 ± 7.47 mg L^{-1}, respectively). Moreover, the concentration of Ag in leaves and in the medium consistently increased with increasing concentrations of AgNPs and $AgNO_3$ (Table 2.2). The fraction of dissolved Ag in control

microcosms and AgNP suspensions was below the detection limit (< 0.01 mg L^{-1}), except for the highest concentration of 100 nm AgNPs, where Ag^{+} released by dissolution was 0.03 ± 0.01 mg L^{-1}.

Table 2.2 - Total silver concentration in oak leaves and in the medium in microcosms supplemented or not with coated (100 nm PVP) and uncoated (35 nm and 50 nm) AgNPs (25 and 200 mg L^{-1}) and $AgNO_3$ (2 and 25 mg L^{-1}) kept at 16 ºC for 28 days. Mean ± SD, n = 3.

Treatments	Concentration (mg L^{-1})	Leaves	Medium
		[Ag] (mg g^{-1})	[Ag] (mg L^{-1})
Control	0	< 0.01 ± 0.00	< 0.01 ± 0.00
35 nm	25	0.18 ± 0.02	2.73 ± 1.09
	200	1.27 ± 0.37	68.78 ± 25.59
50-60 nm	25	0.28 ± 0.03	2.44 ± 0.47
	200	8.25 ± 0.14	70.63 ± 7.47
100 nm PVP	25	0.64 ± 0.50	2.14 ± 1.11
	200	3.33 ± 0.94	58.17 ± 22.55
$AgNO_3$	2	0.01 ± 0.00	0.55 ± 0.04
	25	0.11 ± 0.04	2.98 ± 0.15

2.3.2. Microbial decomposition

After 28 days, oak leaves lost 55 % of its mass in control microcosms. Leaf mass loss was affected by AgNP concentration and size (Fig. 2.1a), and by the interaction between factors (two-way ANOVA, $p=0.008$, $p<0.001$ and $p<0.001$, respectively). Leaf mass loss decreased with the increase in AgNP concentrations for NP sizes of 100 and 50-60 nm. Leaf mass loss was stimulated by 35 nm AgNPs at the highest AgNP concentrations (100 and 200 mg L^{-1}, Bonferroni tests, $p<0.05$).

Fungal biomass on oak leaves was 330 µg ergosterol g^{-1} leaf dry mass in control microcosms. Fungal biomass was affected by AgNP size, but not by concentration (Fig. 2.1b), and an interaction between factors was found (two-way ANOVA, $p\leq0.001$, $p=0.186$ and $p\leq0.001$, respectively). For 200 mg L^{-1} of AgNPs, fungal biomass decreased by almost 50% comparing to the control for all AgNP sizes with the exception of 35 nm AgNPs, where a stimulation was observed (Bonferroni tests, $p<0.05$).

After 28 days in microcosm without stressors, fungal sporulation rate on leaves was 4.5 x 10^5 spores g^{-1} leaf dry mass day^{-1}. Fungal sporulation was strongly inhibited (≥70%) by AgNP exposure (Fig. 2.1c), especially for 100 nm AgNPs (two-way ANOVA, p=0.003 and p≤0.001, respectively; Bonferroni tests, p<0.05). Size and concentration of AgNPs and interaction between factors were significant (p=0.048).

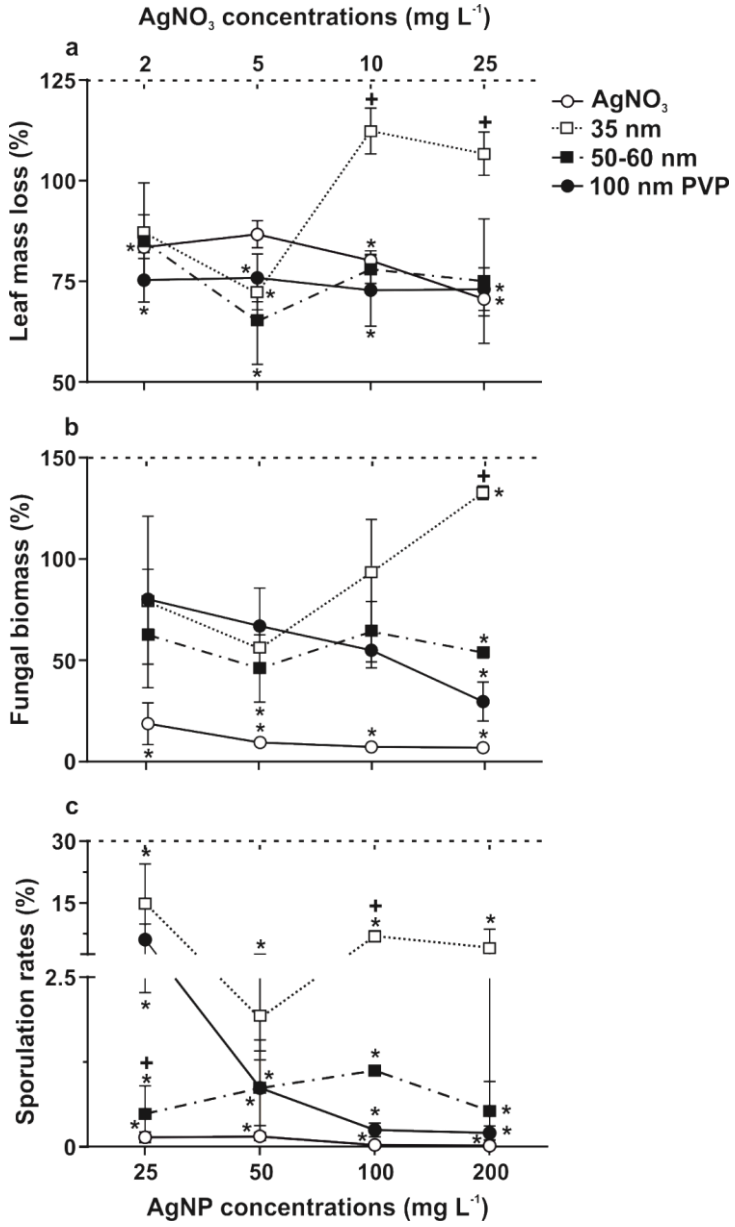

Figure 2.1 - Leaf mass loss (a), fungal biomass (b) and fungal sporulation rates (c) of oak leaves colonized by microbes in the stream and then exposed for 28 days in microcosms to coated (100 nm PVP) and uncoated (35 nm and 50 nm) AgNP and $AgNO_3$. Results are expressed as percentage of the control (n = 3). *, significant effects of AgNPs/$AgNO_3$ concentrations comparing to control (100%); +, significant effect of size at each AgNP concentration (two-way ANOVA, Bonferroni tests, $p < 0.05$).

The activity of the extracellular enzyme β-glucosidase (Fig. 2.2a) was inhibited by increased AgNP concentrations for NP with 100 and 50-60 nm sizes (Bonferroni tests, $p<0.05$). There was an interaction between AgNP concentration and size (two-way ANOVA, $p=0.011$). The phenol-oxidase activity was also inhibited (Fig. 2.2b) by the increase of AgNP concentration of most sizes (Bonferroni tests, $p<0.05$). An interesting result was the stimulatory effect at 100 mg L^{-1} for the 35 nm AgNP.

The increasing $AgNO_3$ concentrations led to an inhibition of all microbial parameters, especially fungal sporulation (Bonferroni tests, $p<0.05$).

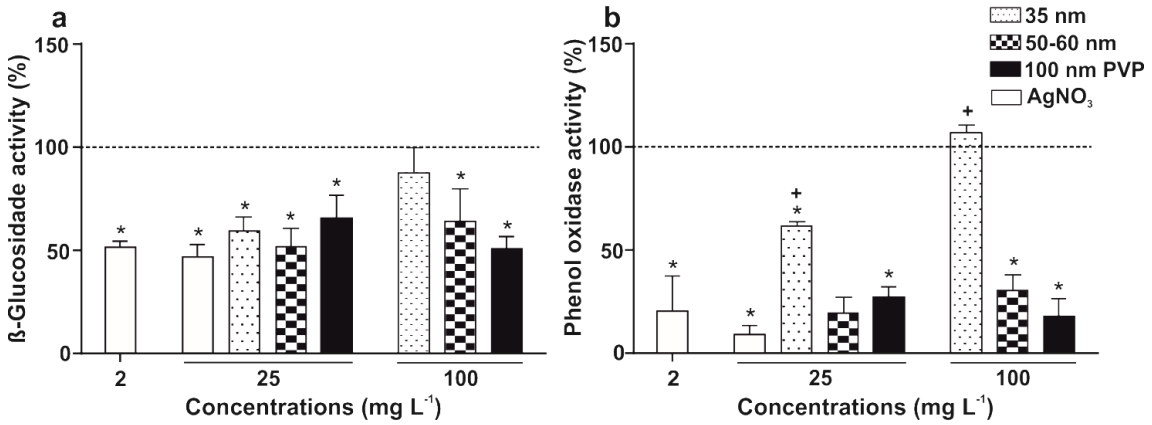

Figure 2.2 - Activities of ß-glucosidase (a), and phenol oxidase (b) on colonizing oak leaves exposed for 28 days in microcosms to coated (100 nm PVP) and uncoated (35 nm and 50 nm) AgNPs (25 and 100 mg L^{-1}) and $AgNO_3$ (2 and 25 mg L^{-1}). Results are expressed as percentage of the control (n = 3). *, significant effects of AgNPs/$AgNO_3$ concentrations comparing to control (100%); +, significant effect of size at each AgNP concentration (two-way ANOVA, Bonferroni tests, $p < 0.05$).

In control microcosms, 11 sporulating fungal taxa were found on decomposing oak leaves (Table S2.1), with *Articulospora tetracladia* (53.3%), followed by *Fontanospora eccentrica* (12.2%), *Dimorphospora follicola* (12.1%) and *Infundibura* sp. (11.1%) the dominant fungal species. In treatments with $AgNO_3$, the number of species was reduced by 80 % at the highest concentrations (Table S2.1). The contribution of *Articulospora tetracladia* decreased in microcosms exposed to AgNPs, whereas *Infundibura* sp. became dominant under exposure to all AgNP sizes. *Fusarium* sp. was only present in microcosms exposed to AgNPs and especially to $AgNO_3$ reaching 74 % of dominance (Table S2.1).

2.3.3. Invertebrate feeding preference

After 5 days of experiment, the shredder *Limnephilus* sp. had a preference for leaves not exposed to AgNPs or $AgNO_3$ (Fig. 2.3). Leaf consumption by the shredders was significantly affected by AgNP size and concentration (two-way ANOVA, $p<0.001$, for both factors), and by $AgNO_3$ (one-way ANOVA, $p<0.001$ (Fig. 2.3).

In general, leaf consumption was inhibited by increasing AgNP concentrations (Bonferroni tests, $p<0.05$), with an exception for NPs with 50-60 nm, where animals preferred leaves exposed to 100 mg L^{-1} of AgNPs (Fig. 3a). Leaf consumption by the shredders was inhibited by 80 % in treatments with leaves exposed to $AgNO_3$ (Bonferroni tests, $p<0.05$) (Fig. 2.3a).

In treatments with leaves exposed to increasing AgNP sizes of each concentration (Fig. 2.3b), shredders demonstrated a preference for leaves exposed to AgNPs with lower size (Bonferroni tests, $p<0.05$) and avoided mainly the 100 nm AgNP (Bonferroni tests, $p<0.05$).

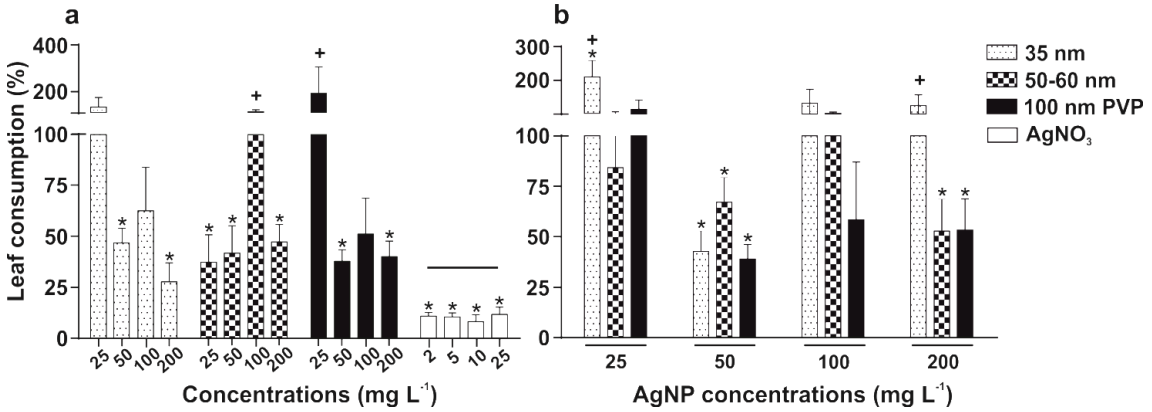

Figure 2.3 – Consumption of oak leaves contaminated with coated (100 nm PVP) and uncoated (35 nm and 50 nm) AgNPs and $AgNO_3$ by the shredder *Limnephilus* sp. during 5 days. For each NP size, animals could choose among leaves contaminated with different concentrations of AgNPs (a). For each NP concentration, animals could choose among leaves contaminated with different sizes of AgNPs (b). Results are expressed as percentage of the control values (n = 4). *, significant effects of AgNPs/$AgNO_3$ concentrations comparing to control (100%); +, significant effect of size at each AgNP concentration (two-way ANOVA, Bonferroni tests, $p < 0.05$).

2.4.Discussion

Our study showed that nano and ionic Ag can be toxic to biota and reduce plant litter decomposition in freshwaters. The impacts of AgNPs were more pronounced on fungal reproduction than on other functional attributes, with a decrease in 85 % of spore production after exposure to the lowest AgNP concentrations. Fungal diversity was also strongly affected by nano and ionic Ag. This may indicate that fungal dispersal and survival in freshwaters may be compromised at longer times with impacts to invertebrate feeding that depends on fungal activity on leaf detritus. Other studies point out that reproduction of aquatic fungi is one of the most sensitive microbial parameters to nano and ionic metals (Pradhan et al., 2011, 2015a). We also observed a shift in species assemblage composition, where some species become dominant in the presence of AgNPs, independently of size or coating, which probably indicates an adaptation of the microbial communities to the stress imposed by nano metals and/or their ionic precursors.

The decrease in the activity of β-glucosidase and phenol oxidase in fungi indicated that these plant litter-degrading enzymes were compromised under nano and ionic Ag stress, and thereby the decomposition activity on leaves was decreased (Sinsabaugh et al., 1994). These results suggest that extracellular enzyme activities might be a good indicator of the stress induced by ionic and nano metals (fungus: Shah et al., 2010; biofilms: Fechner et al., 2012; Gil-Allué et al., 2015).

Other studies support that AgNPs with smaller size are generally more toxic than those with greater size (*Oncorhynchus mykiss*: Scown et al., 2010; *Pseudokirchneriella subcapitata*: Angel et al., 2013; *C. elegans*: Contreras et al., 2014; *Escherichia coli* and *Daphnia magna*: Silva et al., 2014), but here we found the opposite. The larger particle size of AgNPs (100 nm) used in our work contained PVP as dispersant, and particles coated with PVP and citrate are considered well-stabilized AgNPs (less aggregation) compared to the uncoated (Zhao and Wang, 2012; Gomes et al., 2013). Indeed, these NPs were more stable along the experiment comparing to the uncoated smaller AgNPs (35 and 50-60 nm). On the contrary, the uncoated smaller AgNPs (35 nm) were the least stable as demonstrated by the PdI (see Table 1), indicating more aggregation and probably leading to the lower toxicity observed with these AgNPs. Therefore, our study supports that both particle size and coating play a crucial role in AgNP toxicity (Kwok et al., 2016, Siripattanakul-Ratpukdi and Fürhacker, 2014, Zhang et al., 2016).

NPs tend to aggregate onto organic materials due to their small size and highly reactive surface (Holsapple et al., 2005). For the treatments with higher concentrations of 35 nm

AgNPs, leaf mass loss was stimulated, resulting in a higher production of fine particulate organic matter, which in turn could increase AgNP aggregation leading to a decreased toxicity. This probably contributes to explain why treatments with 35 nm AgNPs had a stimulatory effect on fungal biomass. Some studies have shown the role of organic matter in alleviating the toxicity induced by ionic and nano metals (Angel et al., 2013; Pradhan et al., 2015b; Wang et al., 2015). Our results strongly support that the toxicity of AgNPs depends not only on the size and surface but also the state of aggregation influenced by the environmental conditions in which AgNPs are present.

Zeta potential for Ag in natural waters, particularly freshwaters, is generally negative (-20 mV) (Griffitt et al., 2008; Delay et al., 2011), similarly to that measured in the medium at the end of experiment. The highest AgNP concentrations used in our study could have also influenced the stability of AgNPs, by increasing aggregation, lowering electrostatic repulsion between NPs and lowering zeta potential.

Estimated environmental concentration for AgNPs range from ng L^{-1} to µg L^{-1} (Gottschalk et al., 2013) but higher concentrations can be attained during accidental spills and other direct releases that may have significant local effects. In such cases, the ionic Ag released from the NPs might be of significant concern (Garner and Keller, 2014). In fact, Benn and Westerhoff (2008) observed that AgNPs coated socks can release up to 2 mg Ag from 100 to 500 mg of socks after being washed four times.

Although we used concentrations of ionic Ag^+ 10x lower than those of AgNPs, the negative effects of ionic forms were more pronounced compared to their nano forms. In our study, the higher toxicity of $AgNO_3$ compared to AgNPs was probably related to the release of Ag^+ in solution. Less than 1 % of dissolved Ag^+ was found in AgNP suspensions, which means that in our study the toxicity of AgNPs was mainly due to the nano form. AgNPs can interact with the surface of cell membranes, particularly when are less negatively charged (i.e. with lower zeta potential), and can penetrate the cells leading to cellular damage due to direct effects or through Ag^+ released by dissolution from AgNPs. Indeed, ionic and nano forms of silver are reported to induce reactive oxygen species (ROS) in aquatic organisms (Choi et al., 2008; Asharani et al., 2009; Kwok et al., 2016).

AgNP toxicity to invertebrate shredders is generally assumed to occur through waterbone exposure (Blinova et al., 2013; Silva et al., 2014; Ali et al., 2014) and does not take into account the potential impact of AgNP contamination via food. In our study, AgNP size and concentration affected the feeding behavior of the invertebrate shredder *Limnephilus* sp. Freshwater invertebrate shredders prefer to feed on plant litter colonized by microbes,

predominantly fungi, whose activities increase plant litter palatability for shredders (Graça, 2001; Chung and Suberkropp, 2009). The leaves in our study were pre-colonized by microbes, so leaf quality and palatability for shredders might be affected by the impacts of AgNPs on microbial communities. Indeed, the shredders demonstrated preference for leaves exposed to AgNPs of 35 nm where we found a higher fungal diversity and biomass comparing to other treatments. On the other hand, animals avoided the leaves exposed to $AgNO_3$, where fungal diversity and biomass were severely affected. Thus, the stress induced by nano and ionic Ag might have affected the invertebrate shredders directly and/or indirectly due to the effects on microbes.

In our study, we did not observe animal death up to 5 days of leaf exposure to AgNPs or to $AgNO_3$. Post-exposure feeding experiments where invertebrates were exposed to metals (Batista et al., 2012) or metal NPs (Pradhan et al., 2012) did not lead to animal death, but a significant reduction on leaf consumption by the invertebrates was observed. This highlights the use of feeding experiments as a tool for assessing toxicity of contaminants in freshwaters.

Further studies on the analysis of Ag accumulation in the animal body and in fine particulate organic matter (FPOM) produced by the animals would be important, because the binding of metal NPs to FPOM produced by invertebrates can occur and contribute to explain the low recovery of shredding activity after the animals had been released from nano CuO exposure (Pradhan et al., 2015b).

Overall, exposure to nano and ionic Ag inhibited leaf decomposition and microbial activity and diversity, with direct impact in the food choices of the invertebrate shredder *Limnephilus* sp. Fungal reproduction and diversity were the parameters more compromised even at the lowest AgNPs and $AgNO_3$ concentration. Nano and ionic Ag induced sublethal toxicity to the invertebrate shredder. Moreover, the effects depended on particle size and surface coating of AgNPs, independently of the organisms tested in this study.

Our data highlights the importance of considering the physical and chemical properties of NPs when examining the toxicity of the AgNPs to individuals, communities and the processes they drive. Also, our study encourages the use of fungal reproduction and the feeding behavior of invertebrate shredders as endpoints for assessing toxicity of AgNPs in aquatic environments.

To a better evaluation of AgNP impacts it would be important to examine NP interactions with other environmental factors, to further contribute to ascertain the risks of AgNPs in freshwaters.

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Chapter 3

Temperature modulates AgNP impacts on microbial decomposer activity

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Abstract

AgNPs can have toxic effects on aquatic species and compromise important ecosystem processes. AgNP impacts have been the focus of much research, but their effects under different environmental contexts, such as the increase in global temperature are difficult to predict. The aim of this study was to evaluate the interactive effects of AgNPs and temperature on the activity and diversity of microbial decomposers of plant litter in streams. Litter-associated microbial communities were exposed in microcosms to increased concentrations of AgNPs (50 to 75000 µg L^{-1}) and $AgNO_3$ (5 to 7500 µg L^{-1}) and kept for 21 days at 10ºC, 16ºC and 23ºC. Effects of AgNPs and $AgNO_3$ were assessed on leaf mass loss and litter-associated microbial communities by measuring microbial diversity, the activity of fungal extracellular enzymes, and fungal biomass and reproduction. The increase in temperature stimulated leaf mass loss but not fungal biomass and reproduction. Increased AgNP and $AgNO_3$ concentrations inhibited fungal reproduction and diversity, particularly at 23ºC. The activities of the extracellular enzymes phenol oxidase and ß-glucosidase were generally higher at 23ºC. Microbial communities were mainly structured by AgNP and $AgNO_3$ concentrations more than by temperature. The negative effects of nano and ionic Ag on microbial activity were more pronounced at 10 and 23ºC. The behavior of AgNPs was more related to water physical and chemical characteristics (pH) than to temperature. Results highlight the importance of considering different environmental scenarios when examining NP toxicity to freshwater biota and ecosystem processes.

3.1. Introduction

In freshwaters, plant litter decomposition is a key process for the recycling of organic matter (Webster and Meyer, 1997; Gessner et al., 1999) that is mainly degraded by fungi and bacteria and, subsequently, incorporated into food webs (Graça, 2001). Among microbial decomposers, aquatic hyphomycetes play a dominant role in plant litter decomposition by transferring nutrients and energy to higher trophic levels in aquatic food webs (Baldy et al., 2002; Pascoal and Cássio, 2004; Bärlocher and Sridhar, 2014). Plant litter decomposition is sensitive to changes in water quality (metal contamination, Duarte et al., 2008; eutrophication, Pascoal et al., 2003; Pereira et al., 2016), and this ecological process was proposed as a functional measure to assess the health of freshwater ecosystems (Pascoal et al., 2001).

Silver nanoparticles (AgNPs) have been increasingly used over the last decade (The Project on Emerging Nanotechnologies [PEN], 2013) due to their strong antimicrobial properties. The exponentially increasing use of AgNPs has increased the risk that a fraction is released into freshwater ecosystems, where AgNPs and ionic Ag released from NPs can have toxic effects on aquatic species (Fabrega et al., 2011; Navarro et al., 2008a; Pradhan et al., 2012) and compromise key ecological processes, such as organic matter decomposition (Pradhan et al., 2011). The few studies on the impacts of AgNPs on plant litter decomposition have shown that this process is inhibited by exposure to AgNPs probably due to an inhibition of microbial biomass production and sporulation (Pradhan et al., 2011; Tlili et al., 2016).

Interactions between AgNPs and aquatic organisms are poorly understood, but studies indicate that their physical and chemical characteristics, such as NP hydrophobicity or size, can influence NP toxicity (Navarro et al., 2008b; Zhang et. al, 2016). Also, physical and chemical characteristics of the stream water, such as temperature, ionic strength and pH have the potential to affect NP properties (Walters et al., 2013; Siripattanakul-Ratpukdi and Fürhacker, 2014). All these factors can affect aggregation and stabilization of AgNPs and therefore affect their toxicity (Fabrega et al., 2011). It is reported that increase in water temperature led to higher dissolution rates of Ag from AgNPs (Liu and Hurt, 2010; Walters et al., 2013), and some studies suggest that Ag ions released from AgNP dissolution play a major role in the toxicity of AgNPs (Navarro et al., 2008b; Osborne et al., 2013).

Among the environmental variables affecting the behavior of AgNPs, temperature deserves further attention taking into account the global climate change predictions. Current climate models predict an increase in atmospheric temperatures by 2.4–6.4°C by the end of this century, as well as an increase of seasonal temperature extremes in freshwater ecosystems

(International panel of climate change [IPCC], 2013). It is known that temperature directly affects the metabolism of aquatic organisms (Carpenter et al., 1992; Batista et al., 2012; Martínez et al., 2014). Occasional freezing may constrain fungal diversity and their ecological functions, while warming appears to accelerate plant litter decomposition in streams (Fernandes et al., 2009). These responses were accompanied by shifts in the structure, growth and reproduction of aquatic hyphomycete species within assemblages (Fernandes et al., 2009; Ferreira and Chauvet, 2011; Batista et al., 2012; Martínez et al., 2014; Canhoto et al., 2016).

The impacts of AgNPs on freshwaters have been the focus of much research, but their effects under different environmental contexts, such as the expected changes in water temperature, are difficult to predict. Some studies show that temperature induces AgNP aggregation and dissolution, leading to physical and chemical alterations and therefore affecting their toxicity (Oukarroum et al., 2012; Walters et al., 2013; Walters et al., 2016). It is probable that the combined effects of AgNPs and changes in water temperature have strong impacts on the processes in which microbial decomposers are involved, further compromising the functioning of freshwater ecosystems. Moreover, the effects of stressors are usually tested at the level of individual organisms or cells but responses of individual organisms are not necessarily predictive of impacts on higher levels of biological organization such as populations and communities (Van den Brink, 2008; Moe et al., 2012). In an effort to link environmental scenarios and behavioral responses of AgNPs, we evaluated the interactive effects of AgNPs and changes in water temperature at the community level, more specifically on the activity and diversity of microbial decomposers involved in plant litter decomposition in streams. Litter-associated microbial communities were exposed to increasing concentrations of AgNPs and $AgNO_3$ at three temperatures (10ºC, 16ºC and 23ºC). The selection of concentrations used in our study were based on environment predicted concentrations (PECs), which indicate that AgNPs range from ng L^{-1} to µg L^{-1} in surface waters (Gottschalk et al., 2013; Blaser et al., 2008). We expected that water temperature would affect the activity of microbial decomposers and modulate the behavior of AgNPs, by changing AgNP aggregation and/or dissolution of the ionic counterpart and, therefore, altering their toxicity to microbial decomposers.

3.2. Material and methods

3.2.1. Leaf conditioning and sampling site

Alder leaves (*Alnus glutinosa* (L.) Gaertn.) were collected from trees in September 2014, immediately before abscission and dried at room temperature. The leaves were soaked in deionized water and cut into 12 mm diameter discs. Sets of 60 leaf discs were enclosed into fine mesh bags (16x20cm, 0.5 mm mesh) to prevent the access of invertebrate detritivores and submerged in a low-order stream in NW Portugal (Oliveira stream, 41º5863'N 8º22513'W), to allow microbial colonization. A set of three bags was retrieved from the stream after 5 min to determine initial mass of leaf discs. During leaf immersion, conductivity (33 µS cm^{-1}), pH (6.2) and dissolved oxygen concentration (10.5 ± 0.5 mg L^{-1}) were measured in situ using a Multiline F/set 3 no. 400327 (WTW). Stream water samples were collected and transported (4ºC) to the laboratory, and analysed (HACH DR/2000 spectrophotometer, Loveland, CO) to determine the concentrations of nitrate (0.37 mg $N\text{-}NO_3^-$ L^{-1}), nitrite (0.007 mg $N\text{-}NO_2^-$ L^{-1}), ammonia (<0.00 mg $N\text{-}NH_3$ L^{-1}) and phosphate (0.03 mg $P\text{–}PO_4^{3-}$ L^{-1}). The ionic strength measured in the stream water was 2.2 ± 0.1 mmol L^{-1}, total inorganic carbon was 3.6 ± 0.1 mg L^{-1} and dissolved organic carbon was below the detection limit (3 µg L^{-1}) (see in Pradhan et al., 2015).

The riparian vegetation near the stream is mainly composed of alder (*A. glutinosa* Gaertn.), oak (*Quercus robur* L.) and chestnut (*Castanea sativa* Miller), and the stream bottom is composed of rocks and pebbles.

After 11 days, leaf bags were simultaneously retrieved from the stream, placed in a cool box and transported to the laboratory for the microcosm experiment.

3.2.2. Microcosm experiment

Leaf discs from each bag were rinsed with deionized water before being placed in 150 mL Erlenmeyer flasks with 80 mL of sterile stream water. Silver was added to the stream water as citrate coated AgNPs (8 levels: 50 to 75000 µg L^{-1}; NanoSys GmbH, Wolfhalden, Switzerland) or $AgNO_3$ (8 levels: 5 to 7500 µg L^{-1}; >99%, Sigma-Aldrich, St. Louis, MO). Additional microcosms not supplemented with AgNPs or $AgNO_3$ were used as control. Three replicates were set up for each concentration. One set of microcosms was incubated at 16ºC, a temperature commonly found in streams of NW Portugal in autumn, another set at 10ºC and 23ºC to simulate possible scenarios in a climate change (seasonal temperature extremes). All microcosms (total of 153 microcosms) were kept on a shaker (120 rpm) for 21 days and solutions were renewed every 7 days.

The pH of the microcosms was measured at the beginning of the experiment (time 0) and in water from microcosm with microbially conditioned leaves (conditioned water) exposed to 10ºC, 16ºC and 23ºC after 21 days of exposure to AgNPs and $AgNO_3$. At the end of the experiment, leaf mass loss, metal accumulation on leaves, microbial biomass and diversity were determined. For microbial activity on plant litter the following degrading enzymes were measured: ß-glucosidase activity, involved in the last step of cellulose degradation; and phenol oxidase activity, involved in the break down of plant fibers such as lignin.

3.2.3. AgNP characterization and metal analysis

AgNP suspensions of 250, 1000, 10000 and 75000 µg L^{-1} were characterized for NP hydrodynamic diameters and surface charges at the beginning of the experiment (time 0) and also in conditioned water at 10ºC, 16ºC and 23ºC after 21 days of exposure to AgNPs. The hydrodynamic diameter and the surface charge of AgNPs in the suspensions were measured by dynamic light scattering (DLS) using a Zetasizer (Nano ZS, Malvern Instruments Ltd., Worcestershire, UK), and are reported as the mean of three replicate measurements with the corresponding standard deviation.

Total Ag concentration (isotope ^{109}Ag) in the medium and leaves was determined (CACTI, University of Vigo, Spain), after acid digestion, by inductively coupled plasma mass spectrometry (ICP-MS, Thermo Scientific XSERIES 2, USA). The samples were weighted and ignited at 500 ºC (12 h). After cooling, 1 ml of H_2O_2 was added. Then, the samples were dried (100 ºC), ignited again at 500 ºC (1 h) and digested with nitric acid (1 mL, 10%) before quantification of total Ag concentration by ICP-MS. Dissolved Ag from the AgNP suspensions was determined after ultrafiltration (30 min at 3220xg, Megafuge 1.0R, Thermo Scientific Inc., Waltham, MA), using an Ultracel 3k Centrifugal Filter Devices (Amicon Millipore) with a molecular cutoff of 3 kDa (pore size <2 nm) and the Ag concentrations in the filtrate were measured as total Ag.

3.2.4. Leaf decomposition

Leaf discs retrieved from each microcosm before and after the experiment were freeze-dried (Christ alpha 2–4; B. Braun, Melsungen, Germany) to constant mass (± 48 h) and weighed to the nearest 0.01 mg.

3.2.5. Activity of plant litter degrading enzymes

ß-glucosidase activity (EC 3.2.1.21) was analyzed using fluorescent (MUF, methylumbelliferone)-linked artificial substrate (4-Methylumbelliferyl β-D-glucopyranoside, Sigma-Aldrich, Saint Louis, MO, USA). Two colonized leaf discs were placed in 15 mL Falcon vials with 4 mL of sterile stream water, and the substrate was added to a final concentration of 0.3 mM. Controls for water activity (without leaf discs) and fluorescence (without substrate) were performed, and standards of MUF (0–100 μM from Sigma-Aldrich, Saint Louis, MO, USA) were prepared with sterile stream water. Samples, controls and MUF standards were placed on a shaker at temperatures corresponding to each treatment (10, 16 and 23ºC) in darkness for 1 h. Thereafter, glycine buffer (0.05 mM, pH 10.4, 1:1 buffer:sample, volume:volume, 4 mL) was added to each sample to stop the enzyme reaction and maximize MUF fluorescence, and the fluorescence was measured at 455 nm upon excitation at 365 nm (Spectra Max Plus 384, Molecular Devices, Sunny Vale, CA, USA).

Phenol oxidase activity (EC 1.10.3.2 and 1.14.18.1) was measured using L-3,4-dihydroxyphenylalanine (L-DOPA, from Sigma-Aldrich, Saint Louis, MO, USA) according to Sinsabaugh et al. (1994). Incubations (2h) were performed with 5 mM L-DOPA concentration with acetate buffer (pH 5.0). Samples (2 leaf discs) were incubated at temperatures corresponding to each treatment (10, 16 and 23ºC) under shaking in dark. Blanks and standards of L-DOPA (0–10 mM) were also incubated. Absorbance was measured at 460 nm with a fluorimeter (Fluoroskan Ascent FL; Labsystem, Helsinki, Finland). Values were expressed as nanomoles of MUF and L-DOPA per gram of leaves.

3.2.6. Fungal sporulation and biomass

Water samples with conidia released fungi on from decomposing leaves were collected from microcosms after 21 days of exposure to AgNPs and/or $AgNO_3$. Conidial suspensions were fixed with formaldehyde (2% final concentration). Triton X-100 was added to avoid spore adherence to the flasks. Appropriate volumes were filtered (5 mm pore size, Millipore, Billerica, MA) and conidia were stained with 0.05% cotton blue in lactic acid. The conidia of aquatic hyphomycete species were counted (300 conidia per filter) and identified under a light microscope (400x, Leica Biomed, Heerbrugg, Switzerland).

Fungal biomass was quantified measuring ergosterol concentration in leaf tissue, following Gessner (2005). Briefly, lipids from six leaf discs per replicate were extracted in 0.8% of KOH-methanol solution, and were heated to 80ºC for 30 min, and the extracts were purified

by solid-phase extraction. Ergosterol was eluted in isopropanol and quantified by using high-performance liquid chromatography (HPLC) with absorbance detection at 282 nm (column LiChrospher RP18, 25 cm × 4.6 mm, Merck, Darmstadt, Germany; HPLC UltiMate 3000 LC Systems, Thermo Scientific, CA, USA). The extraction efficiency was monitored by using a standard series of ergosterol (Fluka) in isopropanol.

3.2.7. Microbial diversity from DNA fingerprints

Bacterial and fungal assemblages were analysed using denaturing gradient gel electrophoresis (DGGE) after DNA extraction and amplification, following Duarte et al. (2010). DNA was extracted from three leaf discs using an Ultra Clean Soil DNA isolation kit (MoBio Laboratories, Solana Beach, CA). Fungal diversity was assessed using the primer pair ITS3GC/ITS4, which amplifies the ITS2 region of fungal rDNA. Bacterial diversity was assessed using the primer pair 338GC/518, which targets the V3 region of bacterial 16S rDNA.

PCR reactions were carried out in an iCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA), using the following program: initial denaturation at 95ºC for 2 min, followed by 36 cycles of denaturation at 95ºC for 30 s, primer annealing at 55ºC for 30 s and extension at 72ºC for 1 min. The final extension was done at 72ºC for 5 min (Duarte et al., 2010). PCR reagents were purchased from Promega except primers that were purchased from Stabvida.

DGGE analyses were performed using a DCodeTM Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA). Amplification products of 380–400 bp of fungal and 200 bp of bacterial amplified DNA (20 µL per sample) were loaded on 8% (w/v) polyacrylamide gel in 1 × Tris-acetate-EDTA (TAE) with a denaturing gradient from 25 to 65% for fungal DNA and 35 to 70% for bacteria DNA (100% denaturant corresponds to 40% formamide and 7 M urea).

After running the gels (55 V, 56ºC for 16 h), the gels were stained with Midori Green (Grisp) for 10 min on a shaker at 40 rpm. The gel images were captured under UV light in a ChemiDoc XRS (BioRad).

3.2.8. Data Analyses

Two-way analyses of variance (Two-way ANOVA) were used to evaluate the effects of AgNP or $AgNO_3$ concentrations and temperature on leaf mass loss, fungal biomass, fungal reproduction and activity of extracellular enzymes. If an effect was significant, ANOVA

was followed by a post-hoc Bonferroni multi-comparison test (Zar, 2010). Normality of data distribution and homogeneity of variances were checked by Shapiro–Wilk and F tests, respectively.

DGGE gels from each group of microbes, were aligned and the relative intensity of the bands was analyzed with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) and each DGGE band was considered one operational taxonomic unit (OTU).

Canonical correspondence analysis (CCA) was used to determine how fungal and bacterial communities, based on DNA fingerprints, were structured by the variables (temperature and AgNPs/$AgNO_3$ concentration) (Ter Braak and Verdonschot, 1995).

Graph Pad Prism 7 (GraphPad software Inc., San Diego, CA) was used to do the ANOVAs and CCA were done in PAST, version 3.11, for Windows (Copyright Hammer & Harper, Ohio, U.S.; Hammer et al., 2001).

3.3. Results

3.3.1. AgNP characterization and metal analysis

The average particle diameter of AgNPs in fresh water was 26 ± 3 nm for a suspension of 250 μg L^{-1} AgNPs (Fig. 3.1a), and increased to 44 ± 1 nm for a suspension of 75000 μg L^{-1} AgNP. The NP average size had a tendency to increase in the conditioned water exposed to different temperatures, with the exception for the highest concentration (75000 μg L^{-1}), where the size decreased to 30 ± 2 nm at all temperatures. At the beginning of the experiment, AgNPs in fresh water had a zeta-potential of -10 ± 4 at 250 μg L^{-1} (Fig. 3.1b) and increased to -41 ± 4 mV at the highest concentration. The zeta potential increased in conditioned water for 250 μg L^{-1} of AgNPs at all temperatures, but with the increasing concentrations the zeta potential decreased, especially at 23ºC (-38 ± 5 mV at 75000 μg L^{-1}).

The polydispersity index (PdI) of AgNPs in fresh water was below 0.4, indicating monodispersed suspensions at the beginning of the experiment (Fig. 3.1c). The PdI had a tendency to increase in the conditioned water exposed to all temperatures (Fig. 3.1c), and became more stable in suspensions with the highest concentration (75000 μg L^{-1}).

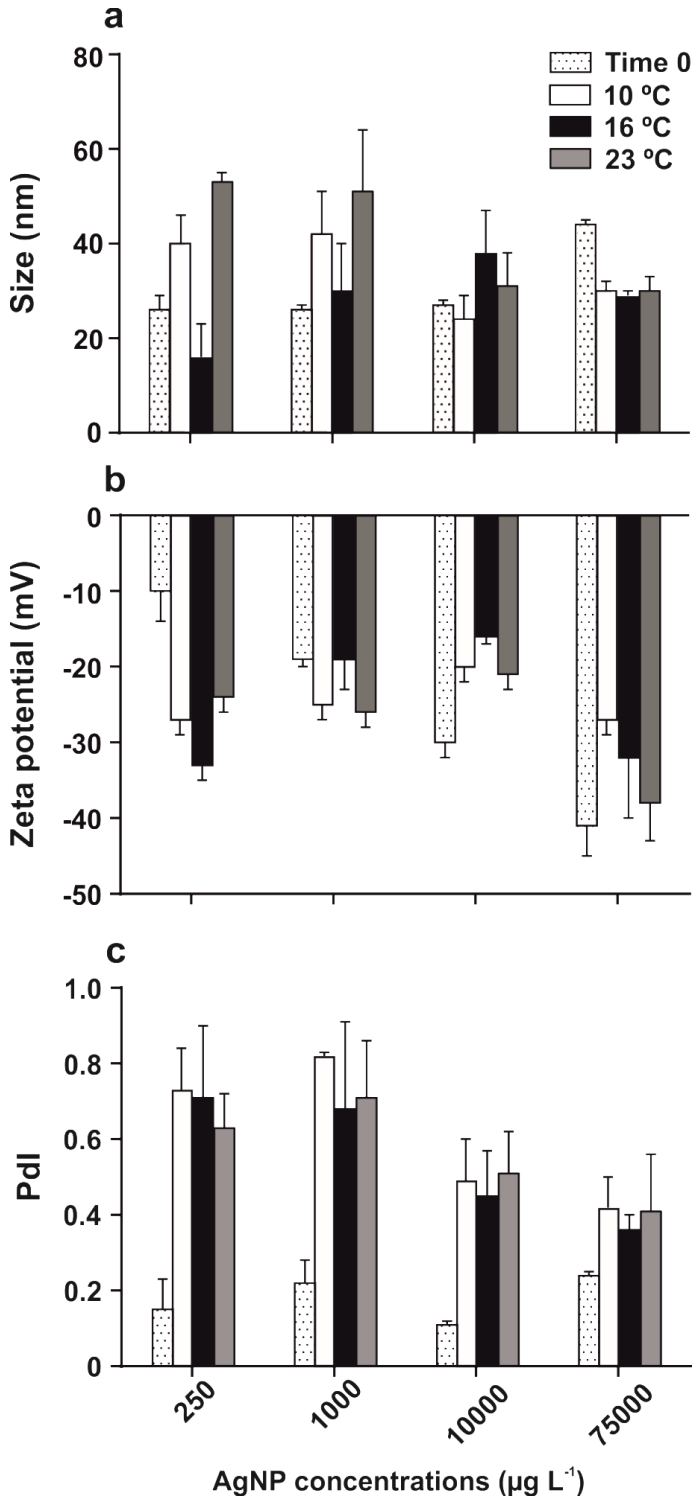

Figure 3.1 - AgNP particle diameter (a), zeta-potential (b) and polydispersity index (PdI) (c) measured by dynamic light scattering (DLS) in AgNP suspensions in fresh water at the beginning of the experiment (time 0) and in conditioned water after 21 days of exposure at 10ºC, 16ºC and 23ºC. Standard deviations refer to three analytical replicates.

The pH in microcosms exposed to AgNPs and $AgNO_3$ were in general higher after 21 days than at the beginning of the experiment (Fig. 3.2). In microcosms exposed to AgNPs, the pH had a tendency to increase with the increasing concentrations after 21 days, and the highest pH values were measured at 23ºC (Fig. 3.2a). In microcosms exposed to $AgNO_3$ the pH values were lower than those in microcosm exposed to AgNPs, and did not change with the concentration (Fig. 3.2b). Again, at 23ºC pH was higher than at lower temperatures.

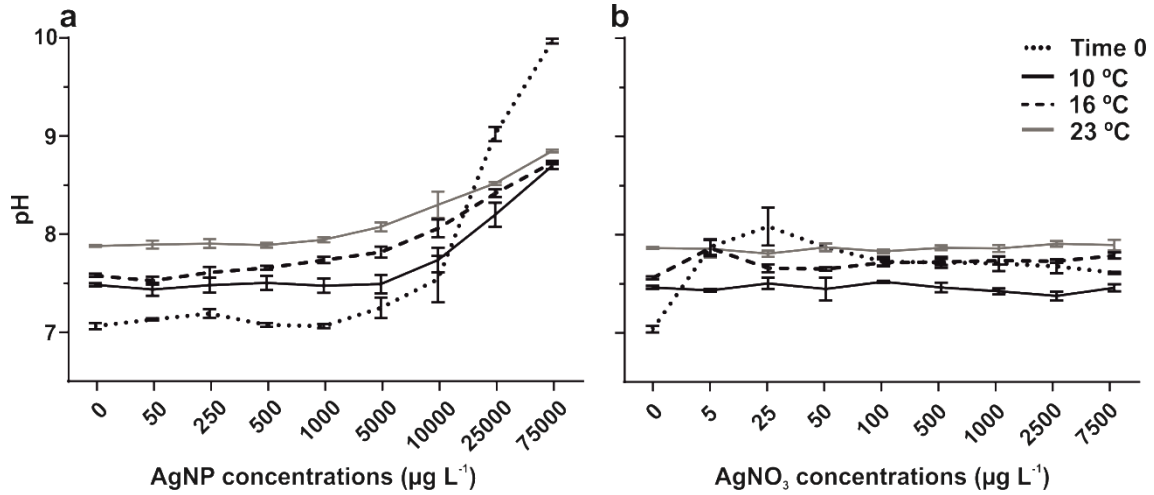

Figure 3.2 – pH measure in fresh water at the beginning of the experiment (time 0) and in conditioned water after 21 days of exposure to AgNPs and $AgNO_3$ at 10ºC, 16ºC and 23ºC. Standard deviations refer to three analytical replicates.

After 21 days, total Ag concentrations in control microcosms exposed to all temperatures were below the detection limit (< 5 µg L^{-1}). The total Ag concentration in the water with AgNPs was between 60% to 100% of the concentration added as nanoparticles (Table 3.1). The accumulation of Ag in the water was more noticed in treatments exposed to 75000 µg L^{-1} of AgNP at 10ºC, while at 23ºC the accumulation of Ag was more prominent on leaves for all treatments. Moreover, the accumulation of Ag on the leaves exposed to $AgNO_3$ was notable, especially because the Ag concentration was 10× lower than that used in the AgNP treatments.

The concentration of dissolved Ag found in 250 and 1000 µg L^{-1} AgNP suspensions was below 10 µg L^{-1} for all temperatures (Table 3.1), while for the highest AgNP concentration the dissolved Ag was lower for 23 ºC (18 ± 2 µg L^{-1}) when comparing to other temperatures (150 and 145 µg L^{-1}, at 10 and 16ºC respectively).

Table 3.1 – Total silver concentration and proportion of dissolved Ag in microcosms exposed or not to AgNPs and $AgNO_3$ at 10ºC, 16ºC and 23ºC after 21 days of experiment. Mean ± SD, n = 3. -, not measured

Temperature	Treatment	($\mu g\ L^{-1}$)	Total Ag		Dissolved Ag
			Leaves	Water	[Ag] ($\mu g\ L^{-1}$)
			[Ag] ($\mu g\ g^{-1}$)	[Ag] ($\mu g\ L^{-1}$)	
10ºC	**Control**	0	< 5	< 5	0
	AgNPs	250	12 ± 2	164 ± 48	< 10
		1000	24 ± 3	1026 ± 66	< 10
		10000	92 ± 2	-	-
		75000	1147 ± 175	71716 ± 993	150 ± 16
	$AgNO_3$	25	10 ± 2	37 ± 4	-
		100	7 ± 1	53 ± 7	-
		1000	110 ± 22	-	-
		7500	525 ± 64	1065 ± 319	-
16 ºC	**Control**	0	< 5	0	0
	AgNPs	250	11 ± 1	136 ± 20	< 10
		1000	18 ± 6	576 ± 36	< 10
		10000	80 ± 10	-	-
		75000	86 ± 50	62580 ± 110	145 ± 22
	$AgNO_3$	25	11 ± 1	35 ± 4	-
		100	15 ± 5	37 ± 5	-
		1000	64 ± 12	-	-
		7500	82 ± 10	671 ± 41	-
23 ºC	**Control**	0	< 5	< 5	0
	AgNP	250	20 ± 3	226 ± 14	< 10
		1000	82 ± 1	767 ± 37	< 10
		10000	143 ± 35	-	-
		75000	2409 ± 500	57456 ± 1340	18 ± 2
	$AgNO_3$	25	19 ± 1	32 ± 5	-
		100	9 ± 2	67 ± 1	-
		1000	59 ± 20	-	-
		7500	1394 ± 200	778 ± 48	-

3.3.2. Microbial diversity from DNA fingerprints

During the whole study, 27 sporulating fungal taxa were found on decomposing alder leaves, but species richness was higher in microcosms incubated at 10ºC. The dominant fungal species was *D. foliicola* followed by *A. tetracladia* and *F. penicillioides* (Table S3.1). Overall, *A. acuminata*, *A. pulchella* and *T. chaetocladium* were absent at high AgNP

and $AgNO_3$ concentrations, whereas *H. lugdunensis* and *F. penicilioides* became dominant. Some species appeared to have preference for higher concentrations of AgNPs and $AgNO_3$, such as *L. aquatica* and *C. aquatica*, respectively (Table S3.1 and S3.2).

The number of species decreased with AgNPs and $AgNO_3$ addition, especially with concentrations higher than 5000 μg L^{-1} and 500 μg L^{-1}, respectively, and this decrease was more pronounced with the increase in temperature from 10ºC to 23ºC. *T. chaetocladium* and *F. curvula* were mainly present at 10ºC, while *T. acuminatus* was mainly present at 23ºC (Table S3.1 and S3.2).

CCA of DNA fingerprints of fungal communities (Fig. 3.3A) were distributed along the gradient of AgNPs and $AgNO_3$ defined by the first axis (79.6% of the total variance), whereas the second axis (12% of the total variance) separated communities according to temperature. CCA of DNA fingerprints of bacterial communities (Fig. 3.3B) showed a pattern similar to that of fungal communities. The variable that most explained community structure was the AgNP and $AgNO_3$ concentrations (66.46%), where the communities exposed to highest concentrations separated from the ones exposed to lower concentrations. Moreover, bacterial communities exposed to 10ºC separated from the ones exposed to higher temperatures.

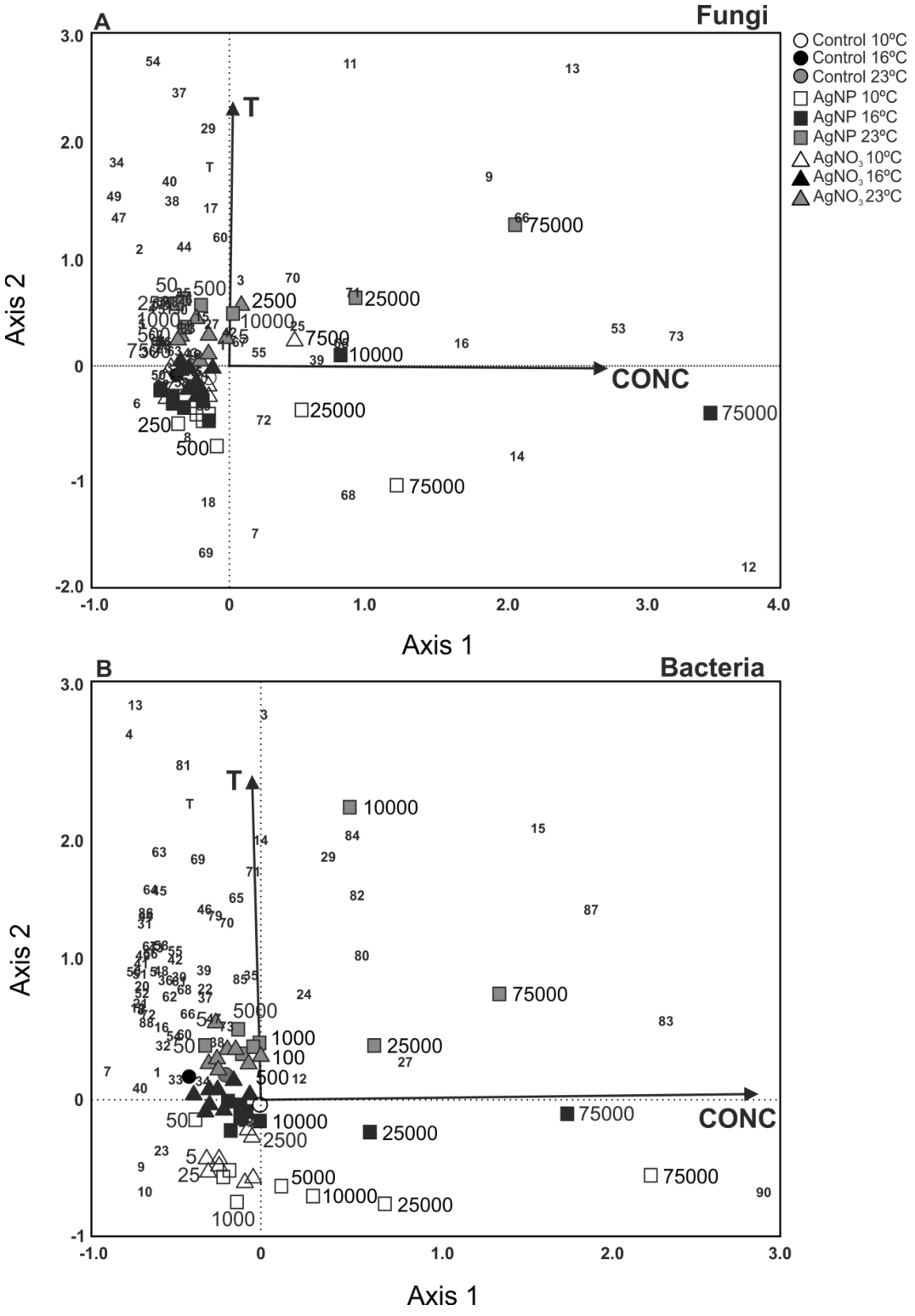

Figure 3.3 - CCA diagrams for ordination of fungal (A) and bacterial (B) communities based on DNA fingerprints of alder leaves colonized in the stream and exposed for 21 days to AgNPs and AgNO3 at 10ºC, 16ºC and 23ºC. Percentages of variance explained by the first two axes were significant (Monte Carlo permutation tests; $P < 0.05$).

3.2.3. Microbial activity and litter decomposition

At 10ºC, alder leaves lost about 23% of their mass after 21 days in control microcosms (without addition of AgNPs or $AgNO_3$). Leaf mass loss was generally higher at 16ºC and 23ºC than at 10ºC. At 10ºC, leaf mass loss was stimulated in microcosms exposed to 75000 µg L^{-1} AgNPs (two-way ANOVA, $p = 0.0386$; Fig. 3.4a), but inhibited in microcosms exposed to 7500 µg L^{-1} $AgNO_3$ (two-way ANOVA, $p = 0.0081$; Fig. 3.4b). The interaction between temperature and AgNPs or $AgNO_3$ was significant (two-way ANOVA, $p = 0.0015$ and $p = 0.0017$, respectively).

After 21 days, fungal biomass on alder leaves ranged from 210 (16ºC) to 260 (10ºC) µg ergosterol g^{-1} leaf dry mass in control microcosms (Fig. 3.4c, d). Fungal biomass was higher at 10ºC than at 16ºC or 23ºC, and was stimulated by the lowest AgNP and $AgNO_3$ concentrations (two-away ANOVA, $p < 0.0001$ for both factors). Fungal biomass was stimulated at 10ºC compared to other temperatures in microcosms exposed to AgNPs at concentrations up to 10000 µg L^{-1} (Bonferroni tests, $p < 0.05$). Fungal biomass was inhibited at the highest concentration of AgNPs mainly at 16ºC and 23ºC (Bonferroni tests, $p < 0.05$). In microcosms exposed to 2500 and 7500 µg L^{-1} of $AgNO_3$ the fungal biomass was strongly inhibited at 23ºC but not at the other temperatures (Bonferroni tests, $p < 0.05$). The interaction between temperature and AgNPs or $AgNO_3$ was significant ($p < 0.0001$).

In microcosms without AgNPs and $AgNO_3$ addition, fungal sporulation rates on leaves were $2.8x10^5$, $1.3x10^5$ and $3.7x10^5$ spores g^{-1} leaf dry mass day^{-1} at 10ºC, 16ºC and 23ºC, respectively (Fig. 3.4e, f). Fungal sporulation was stimulated until 1000 µg L^{-1} AgNPs, mainly at 10ºC (Bonferroni tests, $p < 0.05$), but strongly inhibited at higher AgNP concentrations at all temperatures (two-way ANOVA, $p < 0.0001$ and $p = 0.0023$; Bonferroni tests, $p < 0.05$). The increased concentrations of $AgNO_3$ stimulated fungal sporulation especially at 23ºC, while the decrease in temperature strongly inhibited the sporulation (two-way ANOVA, $p < 0.0001$ for both factors; Bonferroni tests, $p < 0.05$). Temperature and AgNPs or $AgNO_3$ interactions were significant ($p < 0.0001$).

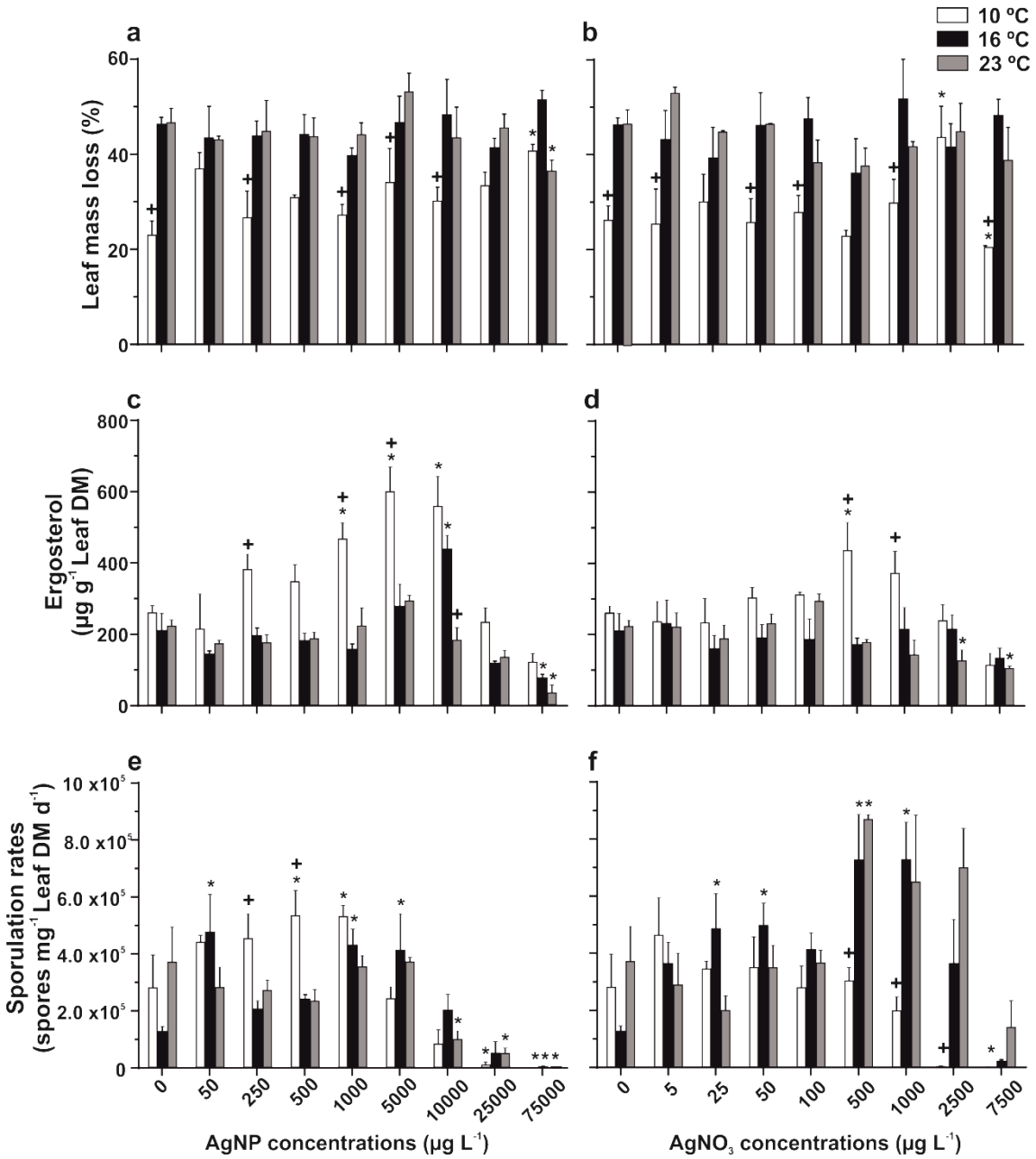

Figure 3.4 – Leaf mass loss (a, b), fungal biomass (c, d), and fungal sporulation rates (e, f) of alder leaves colonized by microbes in the stream and then exposed in microcosms to AgNPs (a, c, e) and $AgNO_3$ (b, d, f) for 21 days at 10 ºC (white bars), 16ºC (black bars) and 23ºC (grey bars). *, significant effects of AgNPs/$AgNO_3$ comparing to control at each temperature; +, significant effect of temperature at each AgNPs/$AgNO_3$ concentration (two-way ANOVA, Bonferroni tests, $p < 0.05$). M ± SEM.

The activity of glucosidase (Fig. 3.5a, b) was affected by AgNP concentrations, but not by temperature (two-way ANOVA, $p < 0.0001$ and $p = 0.7050$, respectively). When leaves were exposed to $AgNO_3$, the activity of glucosidase was stimulated at 23ºC (two-way

ANOVA, $p < 0.0001$ for both factors). The activity of phenol oxidase (Fig. 3.5c, d) was generally higher at 23ºC (two-way ANOVA, $p = 0.0092$ and $p < 0.001$, for AgNPs and $AgNO_3$ treatments). At all temperatures, the increased concentrations of AgNPs and $AgNO_3$ seemed to inhibit the phenol oxidase activity, with an exception at 75000 µg L^{-1} of AgNPs where a stimulatory effect was observed, especially at 16ºC (Bonferroni tests, $p < 0.05$). Interactions between temperature and AgNP or $AgNO_3$ concentrations were significant for both extracellular enzyme activities (two-way ANOVA, $p < 0.0001$; Fig. 3.5).

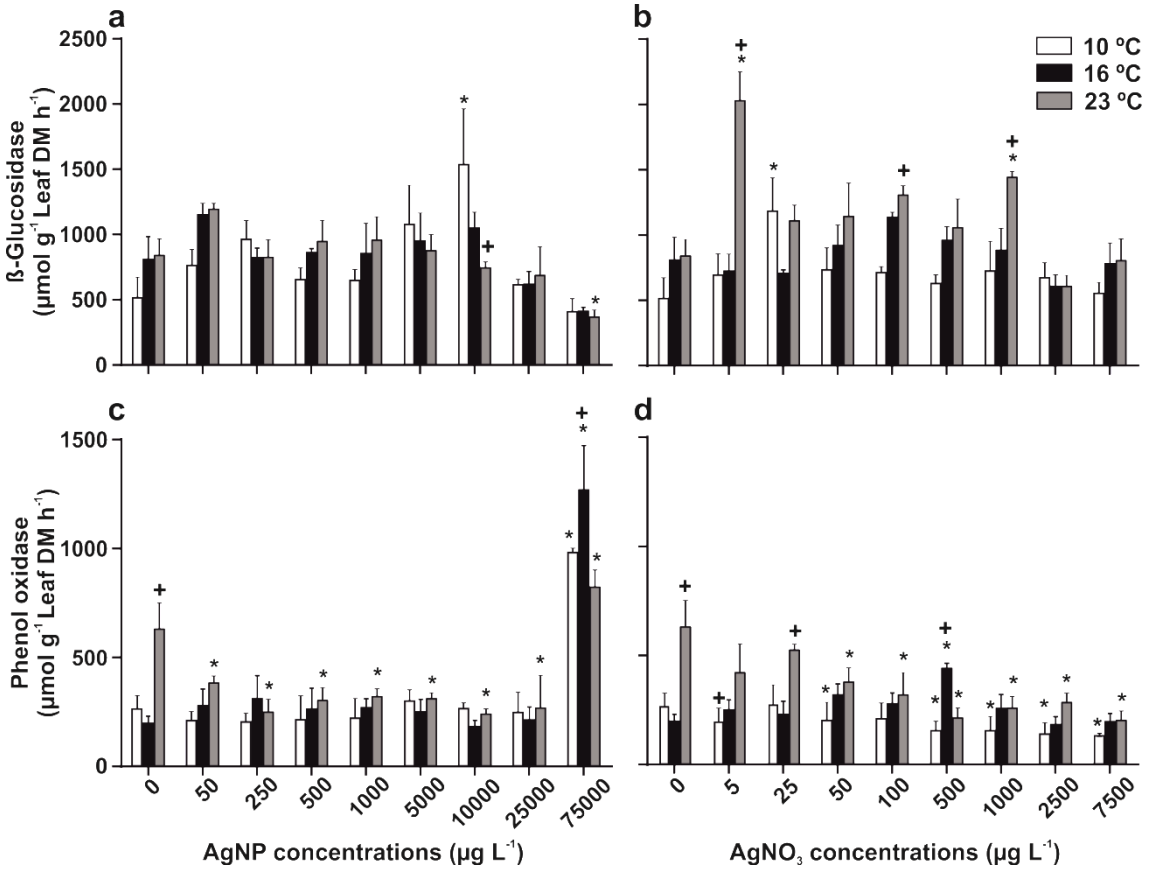

Figure 3.5 – Activities of ß-glucosidase (a, b), and phenol oxidase (c, d) on colonizing alder leaves in microcosms exposed for 21 days to AgNPs (a, c) and $AgNO_3$ (b, d) at 10 ºC (white bars), 16ºC (black bars) and 23ºC (grey bars). *, significant effects of AgNPs/$AgNO_3$ comparing to control at each temperature; +, significant effect of temperature at each AgNPs/$AgNO_3$ concentration (two-way ANOVA, Bonferroni tests, $p < 0.05$). M ± SEM.

3.4. Discussion

Our study addressed some of the challenges in ecotoxicology research by assessing the combined effects of emerging contaminants (AgNPs) under a scenario of changes in

temperature. We succeeded in evaluating the interactive effects between AgNPs and temperature at the community level by examining the activity and diversity of microbial decomposers involved in plant litter decomposition in streams.

In an attempt to predict the effects of global warming, research involving changes in water/air temperature is ongoing. Temperature can affect several biological activities (Brown et al. 2004; Davidson and Janssens 2006), including plant litter decomposition (Martinez et al., 2014) and the associated organisms (Fernandes et al., 2009, 2012; Canhoto et al., 2016). In our study, higher temperatures stimulated leaf decomposition and the activity of leaf degrading enzymes, but not fungal biomass and diversity. The relationship between temperature and metabolism is usually positive (Bergfur and Friberg, 2012, Canhoto et al., 2016), so we expected that higher decomposition in microcosms exposed to 16ºC and 23ºC was ensured by higher fungal biomass (Fernandes et al., 2012; Geraldes et al., 2012; Gonçalves et al., 2013), but fungal biomass and reproduction were higher at 10ºC. This may be partially due to differences in the individual responses of fungal species within communities to temperature changes. If the environmental temperature departs from the species optimum, the fitness of that species, particularly its reproductive output, may decline (Chauvet and Suberkropp, 1998). Another explanation may be related to the enzymatic activity; some species channel the metabolism not to hyphal growth but to increased enzymatic activity (Ferreira and Canhoto, 2015). Indeed, we obtained higher enzymatic activity at 16ºC and 23ºC, even when exposed to high concentrations of AgNPs and $AgNO_3$.

Some authors observed that lower temperatures seem to be more favorable to fungal species (Gonçalves et al., 2013). In our study, fungal species richness was higher in microcosms exposed to 10ºC, and reduced in microcosms exposed at higher temperatures. Some species may have reached their upper thermal tolerance limit, while others may have reached their optimum supporting that fungal species had preference by certain temperature ranges.

The differences in species responses to changes in temperature have consequences for leaf decomposition (Dang et al., 2009; Martínez et al., 2014) if changes in the structure of fungal assemblages affect detritus breakdown by detritivore shredders (Batista et al., 2012), because their activity is enhanced by the presence or dominance of certain fungal species (Canhoto and Graça, 2008; Chung and Suberkropp, 2009).

Independently of the temperature effects, exposure to higher AgNP and $AgNO_3$ concentrations inhibited fungal biomass, reproductive activity and diversity. On the other hand, fungal sporulation was strongly stimulated by lower $AgNO_3$ concentrations (500 and

1000 µg L^{-1}), especially at 16ºC and 23ºC, although fungal diversity was strongly reduced. Fungal sporulation and diversity were sensitive to AgNPs, which agrees with other studies with nano and ionic metals (Pradhan et al., 2011; Tlili et al., 2016). Recent studies have shown that AgNPs can compromise leaf decomposition and affect the associated microbial decomposers (Pradhan et al., 2011; Tlili et al., 2016), but information is scarce and often contradictory because AgNP behavior depend on physical and chemical properties and environmental conditions.

In our study, temperature led to changes in the physical and chemical properties of AgNPs. Even though the AgNPs used in our study were citrate-coated, making them more stable with less probability to aggregate or dissolute (Zhao and Wang, 2012), temperature led to changes in AgNP average particle diameter, especially at lower AgNPs concentrations. Usually, higher leaf decomposition rates increase the release of natural organic matter (DOM) to which AgNPs can aggregate. The binding of NP to natural organic matter is known to affect the NP surface chemistry and NP bioaccumulation (Fabrega et al., 2009; Pradhan et al., 2015).

Leaf decomposition was higher in microcosms exposed to 16ºC and 23ºC, and the increasing AgNP and $AgNO_3$ concentrations did not appear to affect this process. Metabolic demands increase with temperature, and the presence of toxicants leads to an increase in the levels of stress to organisms. In our case, the higher leaf decomposition at higher temperatures during exposure to AgNPs or $AgNO_3$ was probably able to provide the extra energy needed by microbial decomposers to trigger the defense mechanisms against stress. Interesting observation is that at highest AgNP concentrations, the differences in particle size between temperatures became less notable (±30 nm at all temperatures). The fraction of total and dissolved Ag in the water was lower for the highest temperature (23ºC). We expected higher dissolution of AgNPs with the increase in temperature (Liu and Hurt, 2010; Walters et al., 2013), which was the opposite of what we found. Several studies attempted to link dissolution to pH, temperature, AgNP concentration and size. But in our study, we cannot establish a relationship between temperature and pH or Ag dissolution since a similar pH was found at all temperatures at the highest AgNP concentrations.

In this study, the main explanation for the AgNP toxicity is the possible adsorption of AgNPs to the leaves, where fungi are integrated, blocking the release of spores and disabling fungal growth. In fact, accumulation of total Ag was higher in leaves exposed to 10ºC and 23ºC than to 16ºC, especially for higher AgNP concentrations, which supports

the finding above. The negative effects of $AgNO_3$ can also be explained by the notable accumulation of total Ag in the water and especially in the leaves.

A closer look at the results suggests that the behavior of AgNPs may be more related to water characteristics than to temperature. Studies with ZnONPs showed that temperature is a factor less significant compared to pH and electrolyte type for the ZnONPs behavior and fate (Li et al., 2013; Majedi et al., 2014). We would like to emphasize that the release of dissolved Ag from AgNPs was higher only at higher concentrations, regardless the temperature. Moreover, AgNP stability (as changes in PdI and average diameter size) seemed to be more related to the increase in AgNP concentrations than to changes in temperature, despite the differences in PdI and average diameter size of AgNPs at lower concentrations at 10ºC and 23 ºC.

In more sensitive microbial parameters, namely fungal sporulation and diversity, it seems that changes in temperature (10 and 23ºC) enhanced the toxic effects of increasing concentrations of AgNPs. Increases in temperature tend to enhance the toxic effects of some contaminants to aquatic organisms (Heugens et al., 2006; Batista et al., 2012). Although microbial communities seemed to be mainly structured by AgNP and $AgNO_3$ concentrations, we cannot discard the hypothesis that temperature had enhanced the impacts of AgNPs. Temperature changes have the greatest potential to mediate the effects of other more damaging stressors (Jackson et al., 2016).

Research on how temperature modulates AgNP toxicity is scarce, but temperature appears to induce AgNP aggregation and dissolution, leading to physical and chemical alterations and therefore to different toxicity effects (Oukarroum et al., 2012; Walters et al., 2013, 2016). Indeed, our research uncovered that changes in temperature can alter the physical and chemical characteristics of the stream water (pH). These alterations in the water characteristics can be due to DOM released to the water from the higher decomposition rates at higher temperatures. All these factors will influence the toxicity of AgNPs, emphasizing that it is crucial to take into account the interaction of environmental conditions and AgNP physiochemical properties when assessing the toxicity of nano and ionic silver.

Overall, our results showed that the increase in temperature stimulated microbial decomposition of leaf litter and also the activity of leaf degrading enzymes, while low temperature increased fungal biomass and diversity. Increased AgNP and $AgNO_3$ concentrations reduced reproduction and diversity of fungi. The negative effects of nano and ionic Ag on microbial activity on leaves were more pronounced at the lowest and

highest temperatures, suggesting that changes in temperature may promote a more severe threat of AgNPs to aquatic organisms.

In our study, the behavior of AgNPs was more related to water physical and chemical characteristics than to temperature: water pH may have led to changes in the properties of AgNPs, such as dissolution and aggregation. Moreover, AgNPs at environmentally realistic concentrations were toxic towards the microbial decomposers.

Our results highlighted the importance of examining AgNP behavior under different environmental scenarios for NP toxicity, when ascertaining the potential risks of nanomaterials to freshwater biota and ecosystem processes.

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Chapter 4

Impacts of silver nanoparticles and increased temperature on aquatic invertebrate shredders

Abstract

The combined effects of AgNPs and changes in water temperature may have strong impacts on invertebrate shredders with consequences to plant litter decomposition, compromising the functioning of freshwater ecosystems. The aim of this study was to determine how leaf consumption by invertebrate shredders is affected by dietary exposure to AgNPs and whether changes in temperature modulates this relationship. To better understand the mechanisms underlying putative toxicity effects of AgNPs on invertebrates, we further examined the responses of enzymes related to oxidative and neuronal stress at increasing temperatures. To that end, a common species of invertebrate shredder (*Limnephilus* sp.) was allowed to feed on leaves contaminated with AgNPs and $AgNO_3$, and incubated at different temperatures (10ºC, 16ºC and 23ºC). After 5 days the animals were released from the stressors (AgNPs or $AgNO_3$) and allowed to feed on clean leaves. The increase in temperature led to a stimulation of leaf consumption by the shredder, independently the leaves were contaminated or not with AgNPs or $AgNO_3$ and even after the release from the stress. Results from the enzymatic activities demonstrated that AgNP contamination via food can induced oxidative stress in the shredder: the activity of CAT and SOD after the exposure and pos-exposure feeding experiment were associated with the total Ag accumulated in the animal body, while GST activity was strongly associated with treatments exposed to 23ºC. The activity of AChE was stimulated in animals exposed to $AgNO_3$ contaminated leaves at 10ºC and inhibited after the stress release at 16ºC. Overall results suggest that the indirect exposure (via contaminated food) to AgNPs and increased temperatures are influential to the toxicity of metal nanoparticles to invertebrate shredders.

4.1. Introduction

Climate change is an increasingly concern and it is considered one of the major threats to biodiversity, ecosystem functioning and services (Dudgeon et al., 2006; Vörösmarty et al., 2010). The melting of mountain glaciers (Muhlfeld et al., 2011; Gobiet et al., 2014), flow fluctuations (Verdonschot and van den Hoorn, 2010), elevated temperature and salinity, and the deterioration of water quality (Whitehead et al., 2009; Perkins et al., 2010; Woodward et al., 2010) are among the major pressures that many aquatic ecosystems are subjected.

The Intergovernmental Panel on Climate Change (IPCC) has completed assessments covering the evidence, impacts, and mitigation of climate change (IPCC, 2007; 2008). Moreover, the recent IPCC report (2013) anticipates mean global air temperature increases by 2.6–4.8ºC by the end of this century, and it is expected that water temperature follow the air trends (Morrill et al., 2005; Webb and Nobilis, 2007). The small-forest streams, where water temperature is typically low throughout the year, are expected to be highly vulnerable to temperature increases (Carpenter et al., 1992; Dudgeon et al., 2006) with potential implications for the stream biota, particularly on the survival of cold water species (Imholt et al., 2013) including invertebrates (Haidekker and Hering, 2007; Hershkovitz et al., 2015).

Invertebrates play an important role in aquatic systems, supporting several functions such as the processing of organic matter, nutrient cycles, secondary productivity and translocation of materials (Wallace et al., 1996). In low-order forested streams, invertebrate shredders actively participate in the fragmentation of plant material and decomposition of coarse particulate organic matter (Webster and Benfield, 1986), by transforming it into fine particulate organic matter (FPOM), which can be used by filter-feeders and collector-gatherers (Graça, 2001; Graça and Canhoto, 2006). The basal food resources for shredders in streams are dead plant tissues (leaves, twigs, and woody debris) from the riparian vegetation (Anderson and Sedell, 1979; Webster and Benfield, 1986), and leaf fall provides the majority of the annual input of this terrestrial litter (Fisher and Likens, 1973; Webster and Meyer, 1997).

Many species of invertebrates (mayflies, stoneflies and caddisflies) are highly sensitive to temperature changes and other forms of climate change impacts (Haidekker and Hering, 2007; Sauer et al., 2011; Prather et al., 2013; Hershkovitz et al., 2015; Jonsson et al., 2015), and are therefore, more vulnerable to extinction under most future climate scenarios (Hering et al., 2009; Sauer et al., 2011; Domisch et al., 2013; Conti et al., 2014).

It is expected that an increase in water temperature can cause several changes in invertebrates, such as faster initial growth rates, shorter developmental time and smaller size at maturity (Atkinson, 1995; Atkinson and Sibly, 1997). Some authors have reported a higher body mass for aquatic invertebrates at lower then at higher temperatures (Atkinson, 1995; Hogg et al., 1995; Turner and Williams, 2005) and the increase in temperature also change the feeding behavior of invertebrate shredders and the individual body elemental composition (Ferreira et al., 2010). Moreover, these effects are predicted to be stronger for invertebrates inhabiting cold waters when compared to those inhabiting warmer waters (Braune et al., 2008), since biological activities are more temperature limited in cold water environments (Brown et al., 2004). Because invertebrates interact with many trophic groups, from primary producers to top predators, they will have large indirect impacts on ecosystem services under global change (Traill et al., 2010). Climate change can cause shifts in population abundance, geographic distribution and genetics (Parmesan, 2006; Braune et al., 2008; Prather et al., 2013). For example, an increase of 3ºC above ambient temperature, Jonsson et al. (2015) observed a reduction in the number of emergent Chironomidae, while Trichoptera and Ephemeroptera remained unchanged.

The impacts of climate change in freshwater ecosystmens appear to be strongly modulated by other existing pressures, such as eutrophication, and metal pollution (Dudgeon et al., 2006; Perkins et al., 2010; Woodward et al., 2010). Nowadays, silver nanoparticles (AgNP) are widely used in several consumer products mainly because of their antibacterial properties. The rapidly expanding production and use of AgNPs arouse the concern of their release into aquatic ecosystems, where AgNPs and ionic Ag derived from NPs can have toxic effects on many aquatic species (Navarro et al., 2008a; Fabrega et al., 2011; Pradhan et al., 2011: Tlili et al., 2016).

Research about the AgNPs impacts on freshwater invertebrates has received little attention, especially on macroinvertebrate detritivores where the tests are focused on acute effects (Zhao and Wang, 2012a; Völker et al., 2013; Andrei et al., 2016). Sublethal level impacts may allow us to detect changes in animal fitness that are expected to affect their ecological functions (physical and behavior responses: Mehennaoui et al., 2016; or feeding activity: Pradhan et al., 2012, 2015). Concurrently, some studies observed detrimental effects of AgNPs on growth and reproduction of daphnids (Mackevica et al., 2015) and *Chironomus riparius* (Nair et al., 2013; Park et al., 2015), highlighting the need of more quantitative assessments of the chronic and sublethal effects of AgNP exposure. Moreover, the invertebrate behavior as been indicated as a method of assessing NP toxicity despite the

less recognition of this parameter in ecotoxicological studies so far (but see Pradhan et al. 2012, 2015; Ray, 2016).

The activities of antioxidant and neuronal enzymes, such as catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST) or acetylcolinesterase (AChE) in invertebrates are considered the most effective early warming parameters to assess the toxicity of chemicals (Ray, 2016). AgNPs are know to induce oxidative stress in organisms by inducing the production of reactive oxygen species (ROS) (Walters et al., 2016). Organisms have antioxidant defences, which consist mainly of antioxidant enzymes that reduce the damaging effects of peroxides and free radicals that in turn damage macromolecules in cells, including proteins, lipids, and DNA (Halliwell and Gutteridge, 1999). Several studies measured the activity of antioxidant enzymes as a response to AgNPs exposure, and the toxic mechanisms of AgNPs and Ag^{+} can be differentiated by analyzing the responses of these antioxidant enzymes (mussels: Gomes et al., 2015, Gagné et al., 2016; shrimps: Mehennaoui et al., 2016; crayfish: Brittle et al., 2016; crabs: Walters et al., 2016).

It has been often reported that the toxicity of AgNPs depends on their physical and chemical properties, which may vary with environmental factors (Oukarroum et al., 2012; Walters et al., 2013, 2016). The effects of AgNPs and changes in temperature on aquatic ecosystems have been mainly examined independently, but information on the combined mediated effects is lacking. To our knowledge only few studies demonstrated the combined effects of AgNP and temperature on aquatic organisms (alga: Oukarroum et al., 2012; crabs: Walters et al., 2016).

In the previous chapter (chapter 3) we showed that increased AgNPs and $AgNO_3$ concentrations inhibited fungal reproduction and diversity, particularly when the microbial decomposers were exposed to an increase in temperature of about 6ºC. Although AgNP and $AgNO_3$ concentrations have structured microbial communities more than temperature, the hypothesis that temperature can enhance the impacts of AgNPs was not discarded. Changes in microbial decomposer communities can lead to changes in the rates of organic matter decomposition and, therefore, the flow of energy to higher trophic levels.

In this follow-up study, we expected that the combined effects of AgNPs and increased water temperature would have stronger impacts on the processes in which invertebrate shredders are involved (e.g. litter decomposition, nutrient cycling), further compromising the functioning of freshwater ecosystems. To address this question, we determined how leaf consumption by invertebrate shredders is affected by AgNPs and whether changes in

temperature modulate this relationship. To further clarify the mechanisms underlying possible toxic effects of AgNPs on shredders, we examined the responses of antioxidant and neuronal stress enzymes at increasing temperatures. A common species of invertebrate shredders was allowed to feed on leaves contaminated with AgNPs and $AgNO_3$, and incubated at increasing temperatures (10ºC, 16ºC and 23ºC). After 5 days, the animals were released from the stressor (AgNPs and $AgNO_3$) and allowed to feed on clean leaves.

We hypothesized that: i) the increase in temperature would stimulate leaf consumption by the invertebrates but the increase in AgNP concentrations would have an opposite effect; ii) the consumption of leaves contaminated with AgNPs or $AgNO_3$ would induce oxidative and neuronal stress specially at higher concentrations and temperatures; and iii) the release of the invertebrate from the stressors would lead to a recovery in their activity, depending on the temperature that the animal was exposed to.

4.2.Material and methods

4.2.1. Collection of invertebrates and acclimation to the laboratory

Experiments were performed with the invertebrate shredder *Limnephilus* sp. (Tricoptera) of the family Limnephilidae, commonly found in Iberian streams, including low-order streams of North Portugal (Pradhan et al., 2012). The animals were collected at an unpolluted site of the Cávado River (NW Portugal) and acclimated to the laboratory in aerated sterile stream water (121°C, 20 min) at ± 16°C, with a supply of *Alnus glutinosa* (L.) Gaertn. (alder) leaves.

The stream water had the following composition: Ca=1.3 ± 0.3 mg L^{-1}, Cl^-=4.2 ± 0.4 mg L^{-1}, HCO_3=8 ± 0.8 mg L^{-1}, K=0.6 ± 0.1 mg L^{-1}, Na=4.1 ± 0.4 mg L^{-1}, SO_4^-=1.0 ± 0.2 mg L^{-1}) and pH=6.0, with an ionic strength of 2.2 ± 0.1 mmol L^{-1}. Total inorganic carbon in the stream water was 3.6 ± 0.1 mg L^{-1} and dissolved organic carbon was below the detection limit (3 μg L^{-1}).

4.2.2. AgNP characterization and metal analysis

The nanoparticles used were citrate coated AgNPs (NanoSys GmbH, Wolfhalden, Switzerland) and the ionic form was $AgNO_3$ (>99%; Sigma-Aldrich, St. Louis, MO). All the suspensions of AgNPs and $AgNO_3$ were prepared in sterile (121°C, 20 min) stream water. The hydrodynamic diameter and the surface charge of AgNPs in the suspensions was measured by dynamic light scattering (DLS) using a Zetasizer (Nano ZS, Malvern

Instruments Ltd., Worcestershire, UK), and data are reported as the mean of three replicate measurements with the corresponding standard deviation.

Total Ag concentration (isotope ^{109}Ag) in the medium and in the leaves was determined, after acid digestion, by inductively coupled plasma mass spectrometry (ICP-MS, Thermo Scientific XSERIES 2, USA), at the Scientific and Technological Research Assistance Centre (CACTI, University of Vigo, Spain). The total Ag concentration was also determined by the same method in the animal body (larvae) exposed for 5 days to the leaves contaminated with AgNPs and $AgNO_3$ and in the animals after 3 days of release from the toxicants.

4.2.3. Acute lethality tests

Acute lethality tests were performed to evaluate the sensitivity of the invertebrate shredders to AgNPs and $AgNO_3$ and to establish the exposure concentrations for the stress biomarker responses. Invertebrate shredders were starved for 24 h and placed in 250 mL Erlenmeyer flasks with 130 mL of sterile stream water supplemented or not with 6 levels of AgNPs (50 to 75000 µg L^{-1}) or with 6 levels of $AgNO_3$ (5 to 7500 µg L^{-1}). In each flask, 3 animals were placed and 3 replicates were used per concentration, in a total of 9 animals per treatment. The animals were incubated for 96 h at 16ºC, under a 12 h light: 12 h dark photoperiod, and no food was provided during the exposure period. In each 24 h, the number of live and dead animals were determined via visual inspection, and death was assumed when no movement occurred when animals were mechanically stimulated.

4.2.4. Exposure feeding experiment

The animals were kept under starvation for 24 h before exposure to microcosms supplemented with esterile stream water (without AgNPs or $AgNO_3$) and microbially-colonized leaf disks contaminated or not with AgNPs: 5000 µg L^{-1} (>LC_{10}); 10000 µg L^{-1} (LC_{20}-LC_{30}) and 25000 µg L^{-1} (LC_{60}-LC_{80}); and $AgNO_3$: 1000 (LC_{20}-LC_{30}).

Microbial colonization was achieved, as in chapter 3, by immersing leaf disks (12 mm diameter) in the stream for 10 days. Leaf disks were then exposed or not to AgNPs and $AgNO_3$ treatments at 10ºC, 16ºC and 23ºC for 21 days, before being offered to the animals. For each replicate, 2 animals (14.5x1.8 mm length) and 16 leaf disks were placed in 250 mL Erlenmeyer flasks containing 150 mL of sterile stream water. One set of microcosms was incubated at 10ºC, another one at 16 ºC and the other at 23ºC. Six replicates were used per treatment, in a total of 30 microcosms per temperature (total of 90 microcosms).

All microcosms were aerated with air pumps for 5 days and survivorship was registered twice a day during the experiment. At the end of the experiment, half of the replicates were sacrificed (3 replicates per treatment) to quantify leaf consumption and the activity of neuronal and antioxidant enzymes in the shredders. Leaf disks were frozen and lyophilized to a constant weight; and animals were kept at -80ºC to measure the activity of neuronal and antioxidant enzymes. Additionally, the accumulation of Ag in the animal bodies was analyzed.

4.2.5. Post-exposure feeding experiment

After the exposure feeding experiment, animals from a set of 3 replicates per treatment were released from nano and ionic Ag stress: the animals were transferred to microcosms with sterile stream water and were allowed to feed on microbially-colonized leaves (10/12 disks) non-exposed to AgNPs or $AgNO_3$. Animals were kept for 3 days under aeration and photoperiod conditions described in section 4.2.4. Microbial colonization was achieved The leaf disks used in this part of the experiment were colonized by microbial decomposers as mention in chapter 3 and then exposed for 21 days in microcosms without AgNP or $AgNO_3$ addition at 10ºC, 16ºC and 23ºC. At the end of the experiment, leaf disks and the animals were stored and analyzed as mention in section 4.2.4.

4.2.6. Activity of antioxidant and neuronal enzymes in the shredder

The activity of three antioxidant enzymes, glutathione S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD), and the neuronal enzyme, acetylcholine esterase (AChE), were measured. The activities of antioxidant enzymes were assessed in post-mitochondrial supernatant (PMS) from the animal bodies and the activity of AChE was assessed in PMS from the animal head.

4.2.6.1. Preparation of tissue homogenates

Alive animals were retrieved from microcosms, separated from their cases and frozen immediately in liquid nitrogen to prevent cellular metabolism. The animals from each microcosms were homogenized (Ultraturrax IKA, Staufen, Germany) in 100 mM phosphate buffer (containing 2 mM EDTA; pH 7.4) at 11.000 rpm for 25 s. The homogenates were centrifuged (10,000 ×g for 20 min, at 4°C) to separate the PMS. The PMS from body or head tissues was divided into aliquots and stored at -80°C for protein quantification and evaluation of the activities of stress responsive enzymes.

4.2.6.2. Protein quantification

Protein concentration in PMS from the animals was quantified according to Bradford (1976) in a 96-well flat bottom microplate, using bovine serum Albumin (BSA) as standard. Calibration curves were constructed with 0.1, 0.2, 0.5 and 1 mg mL^{-1} and the protein concentration was expressed per wet mass (WM) of animal.

4.2.6.3. Quantification of antioxidant and neuronal enzyme activities

CAT activity was measured according to a modified method of Clairborne (1985). The PMS (6 μL) was added to 994 μL of a reaction mixture containing 0.05 M phosphate buffer (pH 7.0) and 12 mM of H_2O_2. Decrease in absorbance at 240 nm (ε = 43.6 $M^{-1}cm^{-1}$) due to dismutation of hydrogen peroxide (H_2O_2).

GST activity was determined spectrophotometrically at 340 nm (ε = 9.6 mM cm^{-1}) by monitoring the formation of 1-glutathion- 2,4-dinitrobenzene, resulting from the conjugation of the substrate, 1-chloro-2,4-dinitrobenzene (CDNB), with glutathione reduced form (GSH), as described by Habig et al. (1974). The PMS (10 μL) was added to 290 μL of reaction mixture containing 100 mM phosphate buffer (pH 6.5), 1.4 mM GSH and 1.4 mM CDNB. SOD activity was quantified based on its ability to inhibit superoxide radical dependent reactions following the protocol described in the Ransod Kit (Randox Laboratories Limited, Crumlin, UK). AChE activity was determined by the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolysed at 414 nm in a spectrophotometer (ε = 13.6 mM cm^{-1}). The PMS (50 μL) was added to a 250 μL of reaction mixture containing 100 mM phosphate buffer (pH 7.2), 0.25 mg $NaHCO_3$, 0.33 mM 5,5-dithiobis-2-nitrobenzoate and 0.5 mM acetylthiocholine.

All enzymatic activities were calculated in nmoles min^{-1} mg^{-1} protein, except SOD which was expressed as SOD Unit mg^{-1} protein.

4.2.7. Data Analyses

Mortality of invertebrate shredders was recorded, and the concentration inducing death (LC) at 96 h of exposure with the respective 95% C.I. was calculated using PriProbit 1.63 (Sakuma, 1998; http://bru.gmprc.ksu.edu/proj/priprobit/download.asp). Repeated-measures analysis of variance (ANOVA) was used to test the effects of concentrations of

AgNP on the percentage of animal survival in the acute lethality test with matched observations of exposure time (Zar, 2009).

The effects of AgNPs or $AgNO_3$ and temperature on leaf consumption rates and on the activity of antioxidant and neuronal enzymes in *Limnephilus* sp. were tested by two-way ANOVAs (Two-way ANOVA) followed by Bonferroni post-hoc tests (Zar, 2010). No data transformation was done since the data were homoscedastic and had a normal distribution. Graph Pad Prism 5 program (GraphPad software Inc., San Diego, CA) was used for all statistical tests described above.

Leaf consumption by the invertebrates was measured according to Batista et al. (2012), with some modifications: instead of using dry mass of the animals, the calculations were done based on the wet mass (WM) of the animals (as it was needed for the enzymatic assays). Before putting the animals in liquid nitrogen, they were retrieved from the cases, absorbed in tissue paper and weighted.

Relative consumption rates (RCR, g leaf dry mass g^{-1} animal wet mass day^{-1}) was calculated as $RCR = Le/(WMf \times t)$, where Le is the litter dry mass eaten during the elapsed time (t) and WMf is the final wet mass of animals (Batista et al., 2012). Leaf disks were weighed before and after being offered to the invertebrates.

Principal component analysis (PCA) was applied to identify the relationships between AgNPs concentration, $AgNO_3$, temperature, total Ag accumulated in the animal body, and enzymatic activities, after the exposure and the pos-exposure feeding experiment. The PCA was performed in PAST 3.11 for Windows (http://folk.uio.no/ohammer/past; Hammer et al., 2001).

4.3.Results

4.3.1. AgNP charaterization and metal analysis

AgNP charaterization in microcosms where the microbially colonized leaf disks were exposed to AgNPs can be checked in the chapter 3.

After 21 days, total Ag concentrations in leaves non-contaminated with AgNPs and $AgNO_3$ exposed to all temperatures were below the detection limit (< 5 µg L^{-1}). Total Ag concentration in the leaves increased with AgNP concentrations (Table 4.1). The accumulation of Ag in leaves contaminated with AgNPs was higher at 23ºC for all concentrations, while the accumulation of Ag in leaves contaminated with $AgNO_3$ was higher at 10ºC (Bonferroni tests, $p < 0.05$).

The accumulation of Ag in the animals exposed to non-contaminated leaves and in animals exposed to leaves contaminated with $AgNO_3$ was below the detection limit (<0.3 ng mg^{-1} of WM) at all temperatures (Table 4.2). After 5 days of feeding experiment the animals accumulated more Ag in treatments with leaves contaminated with 25000 µg L^{-1} AgNPs at 10ºC, while for the other concentrations the accumulation was higher in animals exposed to 16ºC (Bonferroni tests, $p < 0.05$). After 3 days of toxicant release, the Ag accumulation in the animals was below the detection limit (<0.3 ng mg^{-1} of WM) at all temperatures (data not shown).

Table 4.1 – Total silver concentration on microbially colonized leaves pre-exposed to AgNPs and $AgNO_3$ at 10ºC, 16ºC and 23ºC after 21 days of experiment. Mean ± SD, n = 3.

Temperature	**Treatment**	**(µg L^{-1})**	**Total Ag**
			Leaves
			[Ag] (µg g^{-1})
10ºC	**Control**	0	< 5
	AgNPs	5000	66 ± 2
		10000	92 ± 2
		25000	242 ± 153
	$AgNO_3$	1000	110 ± 22
16 ºC	**Control**	0	< 5
	AgNPs	5000	208 ± 31
		10000	80 ± 10
		25000	495 ± 107
	$AgNO_3$	1000	64 ± 12
23 ºC	**Control**	0	< 5
	AgNP	5000	241 ± 26
		10000	143 ± 35
		25000	592 ± 67
	$AgNO_3$	1000	59 ± 20

Table 4.2 – Total silver concentration (ng mg^{-1} of wet mass of the animal) in the body of the shredder *Limnephilus* sp. allowed to feed on alder leaves contaminated with AgNPs and AgNO3, during 5 days at 10ºC, 16ºC and 23ºC. Mean ± SD, n=3.

Temperature	Treatment	(µg L^{-1})	Total Ag Animal Body [Ag] (ng mg^{-1} of WM)
10ºC	Control	0	< 0.3
	AgNPs	5000	< 0.3
		10000	0.75 ± 0.25
		25000	5.78 ± 0.05
	$AgNO_3$	1000	0.34 ± 0.03
16 ºC	Control	0	< 0.3
	AgNPs	5000	6.16 ± 1.83
		10000	4.35 ± 1.80
		25000	4.53 ± 1.68
	$AgNO_3$	1000	< 0.3
23 ºC	Control	0	< 0.3
	AgNP	5000	< 0.3
		10000	1.09 ± 0.20
		25000	3.35 ± 1.62
	$AgNO_3$	1000	< 0.3

4.3.2. Acute lethal effect of AgNPs on the invertebrate shredder

The exposure of the invertebrate shredder for 96 h to AgNPs and $AgNO_3$ had a significant effect on its survival (repeated-measures ANOVA, $p < 0.05$). The mortality of the shredder increased with increasing concentration of AgNP or $AgNO_3$ and the exposure time (Fig. 4.1). The 96 h LC_{20} and LC_{50} values (95% C.I.) for AgNPs were 7643 (4192-10410) µg L^{-1} and 11475 (8246-18253) µg L^{-1}, respectively. The 96 h LC_{20} and LC_{50} values (95% C.I.) of $AgNO_3$ were 1587 (399-3022) µg L^{-1} and 4664 (2483-24424) µg L^{-1}, respectively.

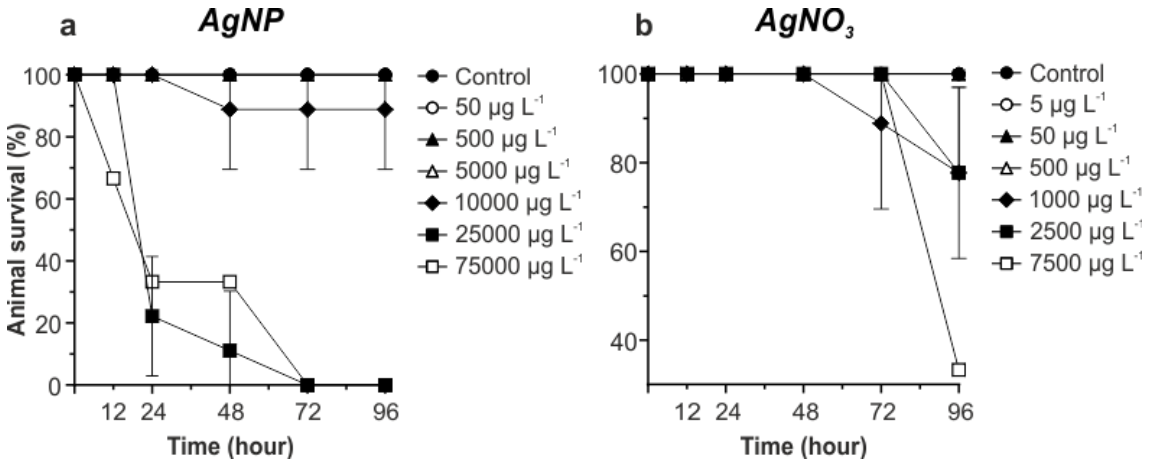

Figure 4.1 - Acute lethal toxicity of AgNPs (a) and $AgNO_3$ (b) to the invertebrate shredder *Limnephilus* sp. with respect to time.

4.3.3. Leaf consumption by invertebrate shredder Limnephilus sp.

4.3.3.1. Exposure feeding experiment

In the absence of AgNPs or $AgNO_3$, the relative consumption rates of alder leaves by the shredder *Limnephilus* sp. were 0.062, 0.066 and 0.101 g leaf dry mass g^{-1} animal wet mass day^{-1} at 10ºC, 16ºC and 23ºC, respectively (Fig. 4.2a). The increased temperature stimulated the consumption rate of the shredder (two-way ANOVA, $p < 0.0001$). Moreover, this stimulation was stronger in treatments at 16ºC where the animals were exposed to leaves contaminated with low AgNP concentrations (5 mg L^{-1}) (two-way ANOVA, $p < 0.0001$), and the interaction between these factors was significant (two-way ANOVA, $p = 0.0016$). At 10ºC, the relative consumption rate was not significantly affected by the exposure to AgNPs or $AgNO_3$ (Bonferroni tests, $p > 0.05$). On the other hand, animals exposed to leaves contaminated with $AgNO_3$ had higher relative consumption rates at 23ºC (Bonferroni tests, $p < 0.05$).

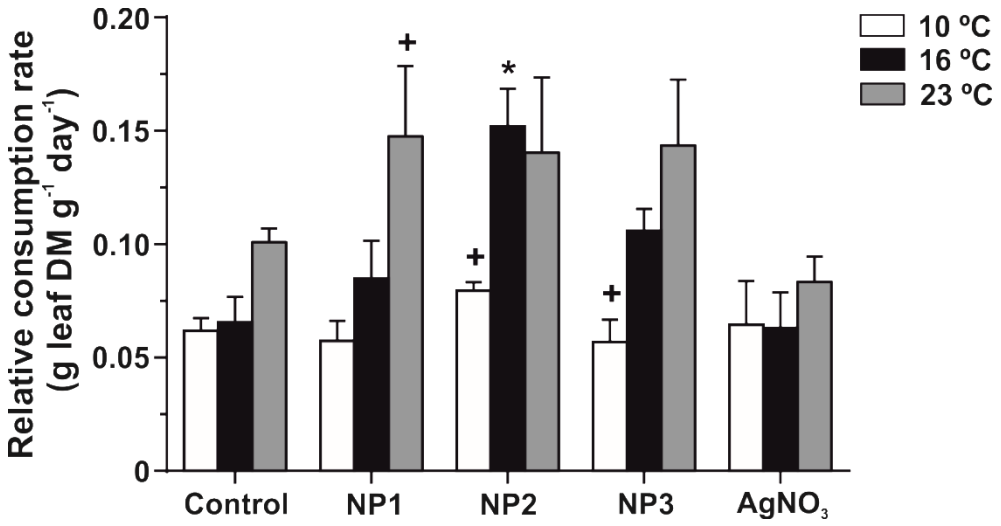

Figure 4.2 - Relative consumption of alder leaves contaminated with AgNPs (control, NP1= 5000 µg L^{-1}, NP2= 10000 µg L^{-1}, NP3= 25000 µg L^{-1}) and $AgNO_3$ (1000 µg L^{-1}), by the shredder *Limnephilus* sp. during 5 days at 10 ºC (white bars), 16ºC (black bars) and 23ºC (grey bars). *, significant effects of AgNPs/$AgNO_3$ comparing to control at each temperature; +, significant effect of temperature at each AgNPs/$AgNO_3$ concentration (two-way ANOVA, Bonferroni tests, $p < 0.05$). M ± SEM.

4.3.3.2. Post-exposure feeding experiment

After the feeding experiment (5 days), animals were released from the toxicants and allowed to feed on non-contaminated leaves for 3 days at the tested temperatures. In control, relative consumption rates by the shredders were strongly stimulated at 23ºC comparing to 10ºC and 16ºC (two way ANOVA, $p < 0.0001$; Fig. 4.3). At 16ºC, the shredders pre-exposed to AgNP and $AgNO_3$ contaminated leaves consumed more than the shredders from the control (Bonferroni tests, $p < 0.05$). Despite the higher relative consumption rate by the shredders at 23ºC there were no differences between AgNP and $AgNO_3$ treatments at 10ºC and 23ºC (Bonferroni tests, $p > 0.05$).

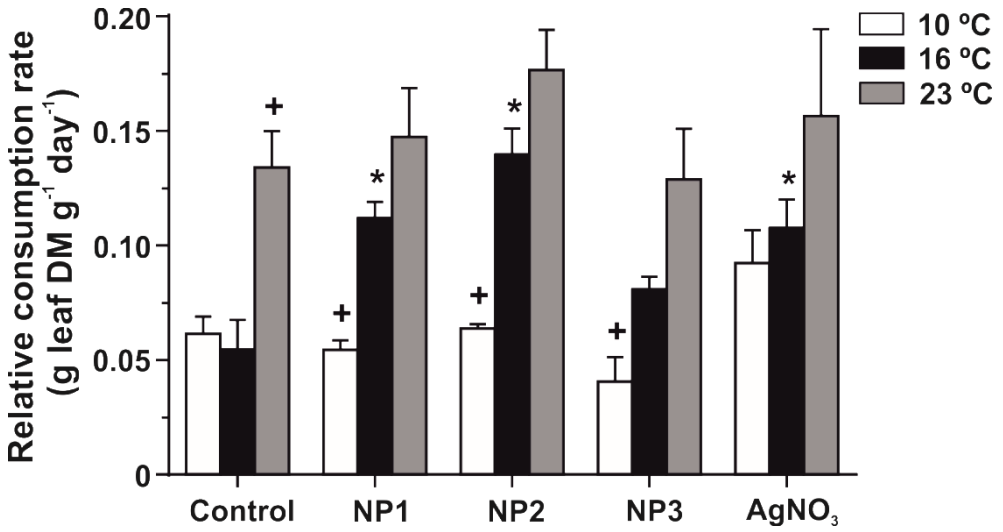

Figure 4.3 - Consumption of alder leaves during 3 days by the shredder *Limnephilus* sp. at 10ºC (white bars), 16ºC (black bars) and 23ºC (grey bars). The animals were previously exposed to contaminated alder leaves with AgNPs (control, NP1= 5000 µg L^{-1}, NP2= 10000 µg L^{-1}, NP3= 25000 µg L^{-1}) and $AgNO_3$ (1000 µg L^{-1}) for 5 days, before being released from the toxicants and supplied with non contaminated leaves. *, significant effects of AgNPs/$AgNO_3$ comparing to control at each temperature; +, significant effect of temperature at each AgNPs/$AgNO_3$ concentration (two-way ANOVA, Bonferroni tests, $p < 0.05$). M ± SEM.

4.3.4. Activity of antioxidant and neuronal enzymes in the shredder Limnephilus sp.

4.3.4.1. Exposure feeding experiment

In control, the protein concentration in the shredder was 18.08, 17.30 and 19.29 µg mg^{-1} WM, at 10ºC, 16ºC and 23ºC, respectively (Table 4.3). The exposure to leaves contaminated with increasing concentrations of AgNPs and $AgNO_3$ did not change the protein concentration in the animal body, independently of the temperature that animals were exposed to (two-way ANOVA, $p = 0.1300$).

Table 4.3 – Protein concentration in the shredder *Limnephilus* sp. allowed to feed on alder leaves contaminated with AgNPs (control, NP1= 5000 µg L^{-1}, NP2= 10000 µg L^{-1}, NP3= 25000 µg L^{-1}) and $AgNO_3$ (1000 µg L^{-1}), during 5 days at 10ºC, 16ºC and 23ºC. Mean ± SD, n=3.

Treatments	Temperature (ºC)	Protein concentration (µg mg^{-1} WM)
Control	10	18.08 ± 1.22
	16	17.30 ± 5.62
	23	19.29 ± 5.11
NP1	10	16.88 ± 5.25
	16	27.04 ± 8.62
	23	17.63 ± 3.44
NP2	10	14.05 ± 0.75
	16	21.95 ± 4.06
	23	14.52 ± 5.13
NP3	10	11.23 ± 2.79
	16	13.55 ± 9.34
	23	15.03 ± 3.22
$AgNO_3$	10	16.95 ± 2.27
	16	15.48 ± 2.17
	23	18.26 ± 3.10

The exposure of shredders to leaves contaminated with AgNPs and $AgNO_3$ for 5 days led to different responses of the antioxidant enzymes (Fig. 4.4). In control, CAT activity in the animal body was 49.57, 57.41 and 46.86 µmol min^{-1} mg^{-1} of protein at 10ºC, 16ºC and 23ºC, respectively (Fig 4.4a). At 10ºC the CAT activity was stimulated only when the animals where fed on leaves contaminated with the highest AgNP concentration (Bonferroni tests, $p < 0.05$), while at 23ºC CAT activity was stimulated only in treatments where the animals where fed on leaves contaminated with $AgNO_3$ (Bonferroni tests, $p < 0.05$). The interaction between factors was significant (two-way ANOVA, $p < 0.0001$).

SOD activity did not differ between treatments at 16ºC and 23ºC, but at 10ºC it was stimulated when animals where fed on leaves contaminated with 10000 µg L^{-1} AgNPs (Bonferroni tests, $p < 0.05$; Fig. 4.4b). Moreover, there was no interaction between factors (two-way ANOVA, $p = 0.13$).

In control, the activity of GST was 5.77, 5.53 and 16.45 86 µmol min^{-1} mg^{-1} of protein at 10ºC, 16ºC and 23ºC, respectively (Fig. 4.4c). The interaction between temperature and concentrations was significant ($p < 0.0001$), with GST activity being stimulated at 10ºC

and 16ºC in animals fed on leaves contaminated with 5000 and 10000 µg L^{-1} AgNPs (Bonferroni tests, $p < 0.05$), respectively.

The activity of AChE in control was higher at 16ºC (two-way ANOVA, $p < 0.0001$). At this temperature, the AChE activity was inhibited in animals exposed to leaves contaminated with AgNPs, while at 10ºC the AChE activity was stimulated in treatments with leaves contaminated with the AgNPs (25000 µg L^{-1}) and $AgNO_3$ (Fig. 4.4d). Moreover, there was an interaction between factors (two-way ANOVA, $p < 0.0001$).

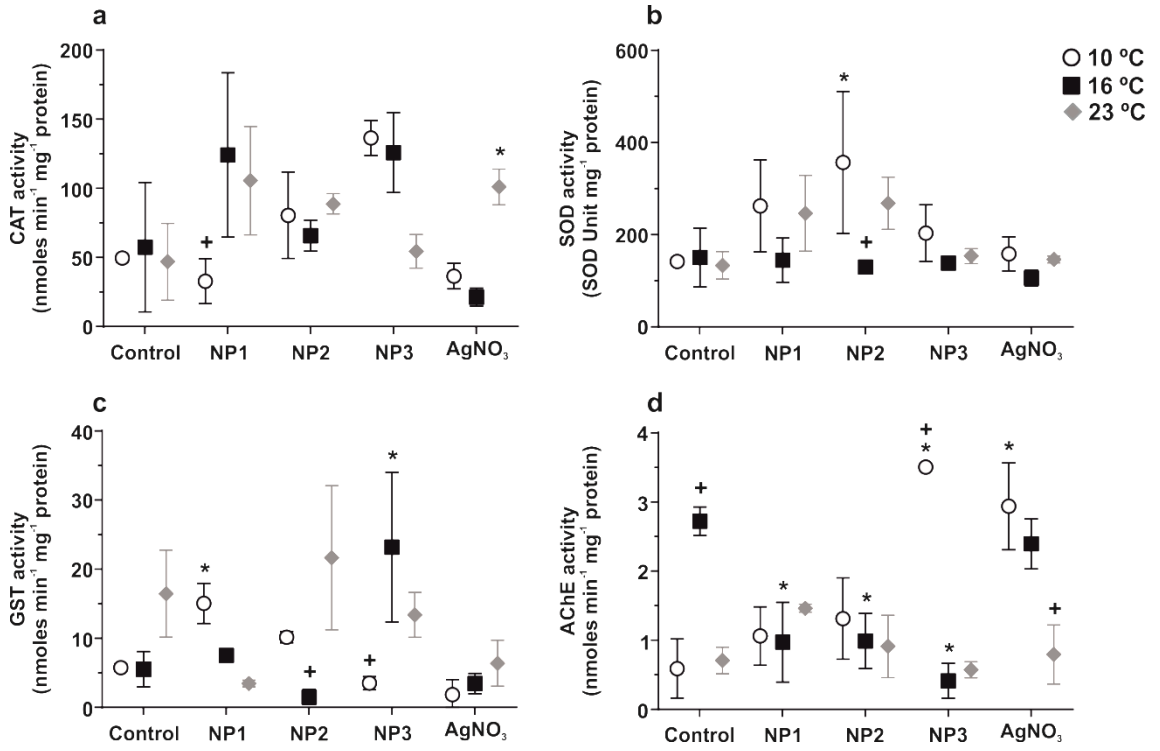

Figure 4.4 - Activities of catalase (CAT; a), superoxide dismutase (SOD; b), glutathione transferase (GST; c), and acetylcholinesterase (AChE; d), in the shredder *Limnephilus* sp. allowed to feed on alder leaves contaminated with AgNPs (control, NP1= 5000 µg L^{-1}, NP2= 10000 µg L^{-1}, NP3= 25000 µg L^{-1}) and $AgNO_3$ (1000 µg L^{-1}), during 5 days at 10ºC, 16ºC and 23ºC. *, significant effects of AgNPs/$AgNO_3$ comparing to control at each temperature; +, significant effect of temperature at each AgNPs/$AgNO_3$ concentration (two-way ANOVA, Bonferroni tests, $p < 0.05$). M ± SEM.

4.3.4.2 Post-exposure feeding experiment

After 3 days of the release from the toxicant, the protein concentrations in the shredder in control treatments were 17.24, 16.91 and 14.59 µg mg^{-1} WM, at 10ºC, 16ºC and 23ºC, respectively (Table 4.4). The protein concentration in the animal didn´t change along the pos-exposure feeding experiment, with an exception of the animals pre-exposed to leaves contaminated with $AgNO_3$: the increased in temperature decreased the protein concentrations when compared to other temperatures (two way ANOVA, $p < 0.0305$).

Table 4.4 – Protein concentration in the shredder *Limnephilus* sp. allowed to feed on alder leaves during 3 days at 10ºC, 16ºC and 23ºC. The animals were previously exposed to contaminated alder leaves with AgNPs (control, NP1= 5000 µg L^{-1}, NP2= 10000 µg L^{-1}, NP3= 25000 µg L^{-1}) and $AgNO_3$ (1000 µg L^{-1}) for 5 days, before being released from the toxicants and supplied with non contaminated leaves. Mean ± SD, n=3.

Treatments	Temperature (ºC)	Protein concentration (µg mg^{-1} WM)
Control	10	17.24 ± 1.93
	16	16.91 ± 6.80
	23	14.59 ± 4.09
NP1	10	17.63 ± 3.29
	16	16.00 ± 4.02
	23	14.32 ± 9.92
NP2	10	16.69 ± 2.79
	16	20.14 ± 1.24
	23	12.23 ± 4.64
NP3	10	17.27 ± 3.67
	16	19.73 ± 3.60
	23	20.19 ± 7.47
$AgNO_3$	10	19.13 ± 2.15
	16	26.80 ± 8.63
	23	12.17 ± 2.57

The responses of antioxidant and neuronal enzymes in the animals that were released from the toxicants and allowed to fed on non contaminated leaves were also analyzed (Fig. 4.5). The release from the toxicants during 3 days also led to different responses of the enzymes, mainly due to differences in temperature (two-way ANOVA, $p < 0.0001$, for all enzymes analyzed).

CAT activity did not differ in animals pre-exposed to contaminated leaves at 10ºC (Bonferroni tests, $p > 0.05$), but at 23ºC the activity was stimulated, especially in animals pre-exposed to leaves contaminated with the highest AgNP concentration (Bonferroni tests, $p < 0.05$; Fig. 4.5a). On the contrary, CAT activity was inibited at 16ºC in animals pre-exposed to leaves contaminated with 10000 μg L^{-1} AgNPs and $AgNO_3$. The interaction between factors was significant (two-way ANOVA, $p = 0.0001$). SOD activity was mainly stimulated at 23ºC in animals pre-exposed to leaves contaminated with the highest AgNP concentrations (Bonferroni tests, $p < 0.05$; Fig. 4.5b). The animals pre-exposed to leaves contaminated with 5000 μg L^{-1} at 16ºC, also showed stimulated SOD activity. There was an interaction between temperature and AgNP concentrations (two-way ANOVA, $p < 0.0001$)

The activity of GST of the animals in control was 11.32, 19.34 and 31.24 μmol min^{-1} mg^{-1} of protein at 10ºC, 16ºC and 23ºC, respectively (Fig. 4.5c). Despite there was no differences between treatments pre exposed to different AgNP and $AgNO_3$ concentrations, the GST activity was highly stimulated at 23ºC (two-way ANOVA, $p = 0.5744$ and $p < 0.0001$, respectively).

After 3 days of release from the toxicants, the animals in control treatments had higher AChE activity at 16ºC (two-way ANOVA, $p < 0.0001$; Fig. 4.5d). The activity of AChE was inibited in the animals exposed to 10ºC (Bonferroni tests, $p < 0.05$). At 16ºC AChE activity was stimulated in the animals pre exposed to AgNP contaminated leaves, but strongly inhibited in animals pre-exposed to $AgNO_3$ contaminated leaves. Moreover, there was interaction between factors (two-way ANOVA, $p < 0.0001$).

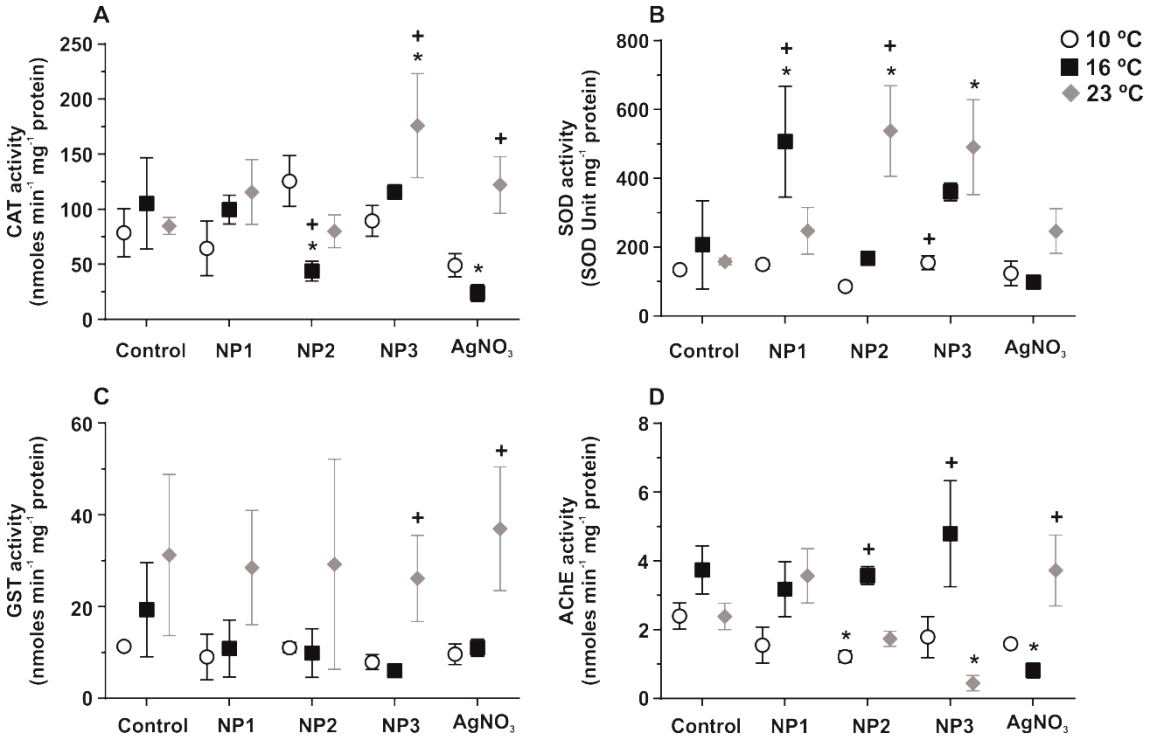

Figure 4.5 - Activities of catalase (CAT; a), superoxide dismutase (SOD; b), glutathione transferase (GST; c), and acetylcholinesterase (AChE; d), in the shredder Limnephilus sp. at 10ºC, 16ºC and 23ºC. The animals were previously exposed to contaminated alder leaves with AgNPs (control, NP1= 5000 µg L^{-1}, NP2= 10000 µg L^{-1}, NP3= 25000 µg L^{-1}) and $AgNO_3$ (1000 µg L^{-1}) for 5 days, before being released from the toxicants and supplied with non contaminated leaves. *, significant effects of AgNPs/$AgNO_3$ comparing to control at each temperature; +, significant effect of temperature at each AgNPs/$AgNO_3$ concentration (two-way ANOVA, Bonferroni tests, $p < 0.05$). M ± SEM.

4.3.4.3 Principal component analysis

The association of the responses of oxidative and neuronal stress enzymes after exposure and pos-exposure feeding experiments, total Ag in the animal body, temperatures and AgNP concentrations and $AgNO_3$ are showed in the principal component analysis (PCA), where PC1 and PC2 explained 30.2% and 18.4% of the total variance, respectively (Fig. 4.6). The PCA showed a clear segregation between treatments exposed to different temperatures along the PC1 based on biomarker responses (Fig. 4.6). The activity of AChE after the feeding exposure was positively associated with the treatments exposed to the lowest temperature (10ºC) along PC1, while the activity of most oxidative stress enzymes were more associated with higher temperatures (Fig. 4.6). With the exception of GST, the

activities of antioxidant enzymes after the exposure feeding experiment were associated with the total Ag accumulated in the animal body along PC2 (Fig. 4.6).

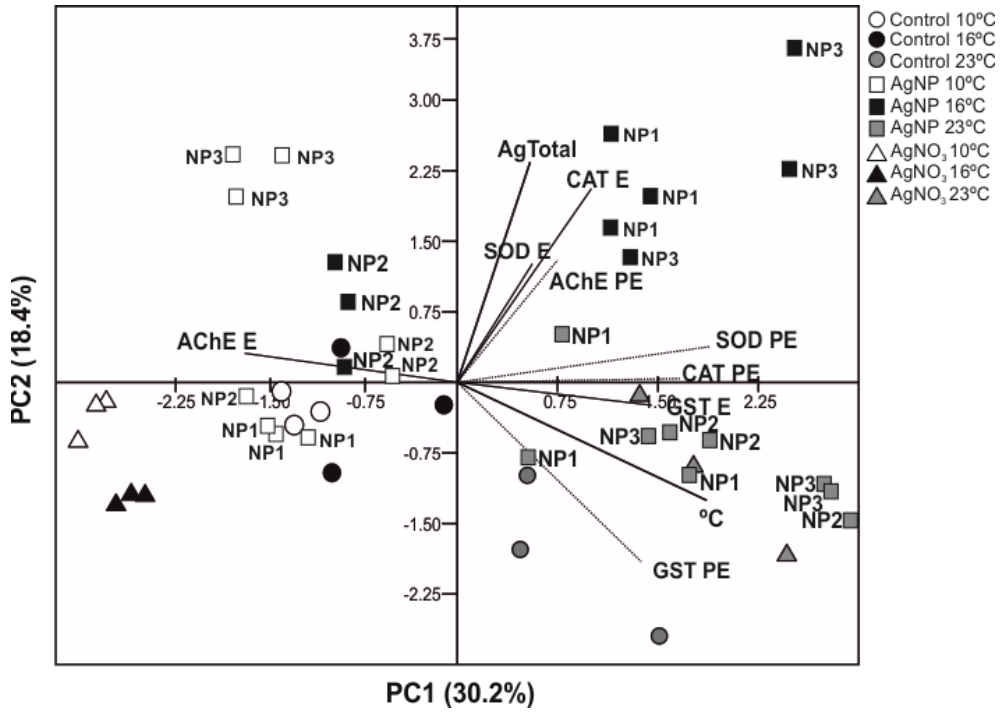

Figure 4.6 - Principal component analysis (PCA) of overall responses of oxidative and neuronal stress biomarkers in the shredder *Limnephilus* sp. allowed to feed on alder leaves contaminated with AgNPs (NP1= 5000 µg L^{-1}, NP2= 10000 µg L^{-1}, NP3= 25000 µg L^{-1}) and $AgNO_3$ (1000 µg L^{-1}), during 5 days (exposure feeding experiment, E) and after 3 days of released from the toxicants and supplied with non contaminated leaves (pos-exposure feeding experiment, PE), at different temperatures (10ºC, 16ºC and 23ºC).

4.4. Discussion

From our knowledge this was the first attempt to assess the interactive effects of temperature and AgNPs on invertebrate shredders. Moreover, the route of AgNP exposure was only via diet and the effects were analyzed when the animals were exposed to contaminated leaves and also after the animals have been released from the stressors when they were supplied with non-contaminated leaves. Ecotoxicological studies have focused primarily on water exposure to test the effects of AgNPs on organisms (Zhao and Wang, 2012; Blinova et al., 2013; Ali et al., 2014), failing to include the toxic effects by diet exposure. Exposure to AgNPs from industry spills or waste-water (Gottschalk et al., 2013) may lead to NPs accumulation or adsorption on organic matter such as leaf litter (Fabrega et al., 2009, Pradhan et al., 2015). So, contamination of invertebrate shredders via food is

highly possible because the main food source for invertebrate shredders is the plant litter present that falls into streams.

In the current study, the acute lethality tests demontrated that the shredder *Limnephilus* sp. can survive until very high concentrations of AgNPs and $AgNO_3$ in the stream water (LC_{50} =11 and 5 mg L^{-1}, respectively). To our knowledge, there is no information besides our study on LC_{50} for this shredder species when exposed to AgNPs or $AgNO_3$. Others reported lower LC_{50} for AgNPs and $AgNO_3$ for other freshwater species, such as *Gammarus fossarum* with a LC_{50} of 835-1000 µg L^{-1}AgNPs (Mehennaoui et al., 2016) and a LC_{50} of 2.2 µg $L^{-1}AgNO_3$ (Arce Funck et al., 2013). The toxicity of $AgNO_3$ is higher than that of AgNPs, as observed in other studies for other freshwater organisms (*Daphnia magna*: Völker et al., 2013; *Gammarus*: Andreï et al., 2016). Despite the high values of LC_{50} observed for the shredder *Limnephilus* sp., the increased concentrations severely affected their survival during the acute toxicity test, and therefore the toxicity of AgNPs and $AgNO_3$ cannot be ignored.

The range of concentrations tested in our study were higher than the predicted environmental concentrations (PECs) for AgNPs in the surface waters that ranged from ng L^{-1} to µg L^{-1} (Mueller and Nowack, 2008; Gottschalk et al., 2013). It is assumed that if NPs are non-toxic at higher concentrations, then they will not be at lower concentrations, which in fact may not be true if longer exposure times are considered and if physical and chemical interactions with organic matter or sediments occur. Organisms living in contaminated ecosystems are likely to feed on contaminated food and it is recognizied that toxicity of AgNPs to aquatic organisms can occur via water and via food (Brinkman and Johnston, 2008). The feeding behaviour of invertebrates is a sensitive endpoint in ecotoxicology for assessing sublethal effects of metals ions (Pestana et al., 2007; Batista et al., 2012) and metal NPs (Buffet et al., 2011; Pradhan et al., 2012, 2015).

In the feeding experiment, the increase in temperature led, as expected, to a stimulation of leaf consumption by the shredders regardless the leaves were contaminated or not with AgNP or $AgNO_3$. Increased temperatures are expected to increase metabolic rates of aquatic organisms (Brown et al., 2004; Clarke, 2006), leading to increases in respiration, growth and development rates (Hogg and Williams, 1996; Atkinson and Sibly, 1997; Harper and Peckarsky, 2006). Increased metabolic rates imply more energy, meaning that the shredders in our study would have consumed more leaves to compensate the energy lost with the metabolism acceleration. A higher consumption rate of leaf litter at higher

temperatures was already observed in invertebrate detritivores (*G. pseudolimnaeus*: Galic and Forbes, 2017; *Limnephilus* sp.: Batista et al., 2012).

Moreover, in our study, the higher consumption rates were not exclusively related to the increased in temperature but also to the presence of leaves contaminated with AgNPs and $AgNO_3$, which was the opposite of what expected. A significant reduction in the feeding rates of clams was observed when fed on diatoms contaminated with 10 µg L^{-1} AgNPs (Buffet et al., 2013). Exposure to toxicants triggers energy consuming defence mechanisms (e.g. detoxification), so organims can increase their intake of energy, to the detriment of other physiological functions (Kooijman et al., 2009), reducing not only their energy reserves but also their fitness through reduced growth and reproduction.

Shredders have the ability to discriminate between fungal species, having preference for certain fungal species (Canhoto and Graça, 2008; Chung and Suberkropp, 2009). Pre-exposure to contaminants can affect leaf quality and palatability for shredders (Batista et al., 2012; Pradhan et al., 2015), reducing their consumption rates, which was not the case in our study. The leaves used in our study were microbially-colonized leaves contaminated with AgNPs and $AgNO_3$ in chapter 3. Although the fungal biomass and species richness was higher in microcosms exposed to 10ºC, some fungal species appeared to have preference for certain temperatures, as well as being present at certain AgNP and $AgNO_3$ concentrations (see chapter 3). Zubrod et al. (2015) also observed preference of *G. fossarum* to leaves exposed to copper over unexposed ones, probably due to changes in fungal community composition.

There are studies reporting a reduction in invertebrate feeding rates after pre-exposure to metals (Moreira et al., 2005; Soares et al., 2005; Batista et al., 2012) or NPs (Pradhan et al., 2015). In our study, the release from toxicants led to higher consumption rates in animals exposed to 23ºC than to other temperatures. Moreover, animals pre-exposed to leaves contaminated with $AgNO_3$ consumed more after released from the stress. In this part of the experiment, leaves were not previously contaminated with AgNPs and $AgNO_3$ and since fungal taxon richness was higher in leaves exposed to 10ºC (see chapter 3) we expected higher consumption rates at these temperature, which was not the case. Despite of the fact that animals were released from AgNPs and $AgNO_3$ contaminated food, they were not released from the temperature stress. Again the metabolism of the animals was accelerated, so they needeed to consume more leaves to compensate their needs in energy. Is seems that higher consumption rates in the pos-exposure experiment was not caused by the release of stress and did not lead to a recovery of animals activity, but the high

temperatures used in our study may have led to high levels of stress in the animals. In a global warming scenario, we may expected an increase in leaf litter decomposition in the presence of shredders, which may result into a reduction of food suply in streams, affecting negatively higher trophic levels in aquatic ecosystems (Moghadam and Zimmer, 2016).

Despite the exposure to AgNP or $AgNO_3$ contaminated leaves did not lead to a reduction in the feeding rates of the shredder, it does not mean that these contaminants are not toxic. In a study with *D. magna*, Zhao and Wang (2011) demonstrated that despite only 10% mortality was observed after diet exposure to $AgNO_3$, the reproductive performance was significantly affected by dietary Ag exposure. The complementarity with the analysis of antioxidant and neuronal enzymes in the animals helped us to understand that higher consumption rates were associated with a strong response to AgNPs, at the cellular level as proved by our results from enzimatic activities.

In our study, the activity of antioxidant enzymes in the shredder *Limnephilus* sp. showed different responses according to the concentrations of AgNPs and $AgNO_3$ and the temperature tested. Oxidative stress is one of the most common effects of nanotoxicity reported in many studies assessing toxicity mainly through water exposure (Klaine et al., 2008; Buffet et al., 2011; Ali et al., 2014; Pradhan et al., 2016). In our study, the oxidative stress was observed also by dietary exposure. Buffet et al. (2013) highlighted that the responses of antioxidant enzymes, similar to those assessed in our study (GST, CAT and SOD), were more important after dietary exposure than after water exposure to AgNPs in marine bivalves.

In our study, the stimulation of SOD and CAT in animals exposed to leaves contaminated with the highest AgNP concentration at 10ºC suggests the production of superoxide anions triggered by AgNPs and the role of SOD in early defense against oxidative stress at cellular level. SOD catalyse the dismutation of the superoxide radical O_2 into oxygen and H_2O_2, which is decomposed by CAT into O_2 and water (Company et al., 2008; Villarreal et al., 2014). Walters et al. (2016) also observed stimulation of CAT and SOD in the crabs *Potamonautes perlatus* after waterborne exposure to AgNPs. Antioxidant enzymes such as SOD and CAT may be induced in mild oxidative stress conditions as a compensatory response. However, an over-production of ROS might overwhelm the detoxifying and antioxidant mechanisms, leading to significant oxidative damage and a loss of compensatory mechanisms, thereby suppressing the activities of the antioxidant enzymes (Zhang et al., 2004).

CAT stimulation at 23ºC in animals pre-exposed to leaves contaminated with the highest AgNP concentration suggested that the increase in antioxidant defenses were due to enhanced oxygen free radicals production, which could stimulate antioxidant activities (Torres et al., 2002) to cope with this increased oxidative stress and protect the cells from damage (Ali et al., 2014).

The increased GST activity in animals that were fed on contaminated leaves with the higher AgNP concentration at 16 and 23ºC, suggested an increased intracellular accumulation of ROS. On the contrary, a significant reduction of GST was observed in the freshwater pulmonate snail *Lymnaea luteola* when exposed to 36 µg L^{-1} AgNPs by waterborne exposure (Ali et al., 2014). A higher GST activity implies a greater detoxification capacity (Pinho et al., 2005) or conjugation of oxidative products. In our study, this was more pronounced in the animals after stress release at 23ºC, which indicates that temperature can trigger oxidative stress in the animals.

In our study, the neuronal functions were compromised in shredders that fed on leaves contaminated with AgNPs at 16ºC, as the AChE activity was inhibited in those animals. The shredders had their neuronal functions still affected after the release from the toxicants, mainly at 23ºC. AChE plays an essential role in cholinergic neurotransmission in the central nervous system and neuromuscular junctions (Vieira et al., 2009) and it is one of the principal enzymes that are considered for toxicity screening (Ray, 2016).

We expected that higher temperatures led to a less production of ROS, since the dissolution of oxygen is generally lower at higher temperatures, so oxidative stress responses are usually inversely correlated with temperature (Vinagre et al., 2012). However, after release of the toxicants, the oxidative stress responses in the shredders were generally higher at 16ºC and 23ºC, as reported by Walters et al. (2016) in crabs exposed to high stress (i.e., 100 µg/mL AgNP at 28°C). As mention above, at higher temperatures the animal metabolism acceleration implies more energy, which may lead to higher O_2 consumption. Our results support that these stress oxidative enzymes are highly sensitive to temperature, likely due to temperature-induced ROS production. Several studies provided evidence of a relationship between thermal stress response and oxidative stress responses in a fish (Madeira et al., 2013); and strong correlations between SOD, GST and CAT activities in crabs exposed to AgNPs at 28ºC (Walters et al., 2016)

The total Ag found in the animal bodies after the feeding experiment under AgNPs or $AgNO_3$ exposure was very low (ng mg^{-1} of WM), when comparing to the concentrations found in contaminated leaves with AgNPs or $AgNO_3$. Several authours reported Ag

accumulation in freshwater invertebrates not only after waterborne but also dietary exposure (Croteau et al., 2011; McTeer et al., 2014; Ribeiro et al., 2016). The contaminated food was added in the water for a period of time and, inevitably, NPs or Ag^{+} could be released from the NPs from the food to the water, and the degree of such release will depend on the location of the metal. Moreover, the accumulation of NPs in aquatic organisms dependent on both the uptake (ingestion or adsorption) and the elimination (detoxification) of the NPs out of the organism, such as faecal production (Zhao and Wang, 2012; Andreï et al., 2016). Our inability to detect Ag in the animal body after 3 days of stress release, suggest that in 3 days the animals could have eliminated Ag accumulated maybe by feces excretion.

The PCA demonstrated that antioxidant enzymes were correlated to the total Ag accumulation in the animal bodies, with the exception of GST that was more correlated with the treatments exposed to higher temperatures (23ºC). The exposure to contaminated leaves with AgNPs could result in the accumulation of Ag in the animals, triggering enzymatic responses at a cellular level. Being a major food source for predators, shredders can be a potential vector of bioaccumulation in the food web, and the elimination of AgNPs by the production of fine particulate organic matter, including feces, can lead to toxicity to other detritivores (e.g. collectors).

In the feeding experiment, the increases in temperature led to a stimulation of leaf consumption by the shredder *Limnephilus* sp. Moreover, the higher consumption rates were not exclusively related to the increase in temperature but also to the presence of leaves contaminated with AgNPs or $AgNO_3$. The animal release from the toxicants did not change the feeding activity, because after 3 days in the presence of non-contaminated leaves, shredders consumed more at 16ºC and 23ºC.

The most significant results were observed for the antioxidant and neuronal enzymes: results from the enzymatic activity demonstrated that AgNP contamination via food could induce oxidative stress in the shredders. The absence of negative effects in the consumption rates by the shredders can be misleading, because results from the enzymatic activity suggests that AgNPs could induce toxicity indirectly via food. Shredders have a dominant role in leaf litter decomposition by transforming leaf material into fine particles that are food resources to other invertebrate detritivores, so that contaminants can be transported through the detritus food chain compromising higher trophic levels (Cushing et al., 1993).

Moreover, the effects on consumption rates and enzymes are more related to the higher temperatures, highlighting the importance of addressing the effects of increased temperature on aquatic organism already sensitive to contaminants.

Finally, it may be necessary to develop ecological risk assessment procedures that take into account the toxicity of AgNPs via food: the dietary exposure should be incorporated into the water quality criteria. The mechanisms of dietary exposure are poorly investigated, especially when AgNP is involved, and the few studies investigating this pathway demonstrated a strong difference from waterborne exposure.

This work brings new insights to the understanding of environmental impacts associated with the exposure to AgNP via food, providing evidence that indirect exposure of AgNPs and increased temperature are influential to assess the toxicity of AgNPs towards the shredder *Limnephilus* sp.

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Chapter 5

Pollution-induced community tolerance of microbial decomposers to silver nanoparticles

Abstract

The production and use of silver nanoparticles (AgNPs) have significantly grown over the last decade, increasing the risk that a fraction is transported to freshwater ecosystems, where AgNPs and released ionic silver (Ag) can impact communities and processes. Plant litter decomposition is an important ecosystem process in which both fungi and bacteria contribute, with different microbial species varying in their sensitivities to pollutants. The goal of this study was to determine whether chronic exposure of microbial decomposers to low concentrations of nano or ionic Ag could induce shifts in bacterial and fungal communities, leading to an increased tolerance towards AgNPs and $AgNO_3$. Tolerance acquisition was measured via short-term bioassays using several microbial endpoints: fungal sporulation, bacterial production, microbial respiration and the activity of leucine aminopeptidase. DNA fingerprints of microbial communities showed that the structure of fungal and bacterial communities exposed to the highest AgNP concentrations (100 and 200 µg L^{-1}) grouped together and separated from the control community. Communities exposed to AgNPs and $AgNO_3$ showed higher tolerance as revealed by the effects on fungal sporulation and bacterial biomass production. These results suggested that the application of pollution-induced community tolerance (PICT) to microbial decomposer communities might be a powerful tool for risk assessment of AgNP in freshwaters.

5.1.Introduction

The production and use of silver nanoparticles (AgNPs) have been exponentially growing over the last decade, increasing the probability that a fraction of AgNPs is transported to wastewaters and ultimately to rivers and coastal waters. Aquatic environment is susceptible to AgNP exposure and aquatic organisms can represent a target group for AgNP uptake and effects (Moore, 2006; Canesi et al., 2011). Although AgNPs are known to be toxic to many species (Navarro et al., 2008a; Fabrega et al., 2011; Pradhan et al., 2011 and 2012), there is still debate on which extent toxicity is due to the nanoparticle itself or the associated ionic form. Some studies suggest that silver ions released from AgNP dissolution play a major role in the toxicity of AgNPs (Osborne et al., 2013), while others report that toxicity is caused by intrinsic AgNP properties related to their high surface area and reactivity (Fabrega et al., 2009; Tlili et al., 2016a).

The effects of AgNPs have been mainly studied in single organisms (Handy et al., 2012; Dorobantu et al., 2015; Andreï et al., 2016), which do not reflect the impacts to the entire community and associated ecological processes. Therefore, the complexity of natural communities and ecosystems should be considered in ecotoxicological studies on the environmental effects of AgNPs.

Plant-litter decomposition is a key process in freshwater ecosystems, which depends on the activity of microbial decomposers, namely fungi and bacteria, and invertebrate shredders (Graça, 2001). Fungi, particularly aquatic hyphomycetes, have been identified as dominant microbial decomposers, while bacteria are recognized to play a role after partial breakdown of plant material (Pascoal and Cássio, 2004; Gessner et al., 2007).

The few studies focused on the impacts of AgNPs on plant litter decomposition proved not only the leaf decomposition decreased with exposure to AgNPs but also microbial biomass and sporulation rates are inhibited by AgNPs (Pradhan et al., 2011; Tlili et al., 2016a). Aquatic hyphomycete species have been found in metal polluted streams (Pascoal and Cássio, 2004) and, thus, they can develop adaptive mechanisms toward tolerance against metals (Guimarães-Soares et al., 2007; Azevedo et al., 2009). Data from the literature indicate a greater tolerance to metals in fungi isolated from metal-polluted sites (Miersch et al., 1997), and a recent study by Pradhan et al. (2015a) demonstrated that fungi isolated from metal polluted streams are more resistant to CuONPs than fungi isolated from nonpolluted streams. These studies suggest that tolerance acquisition by microbial communities can be used as specific indicator of nano and ionic metal pollution in freshwater ecosystems.

Microbial communities are composed of different species with variable sensitivities to stressors, which form the basis of the pollution-induced community tolerance (PICT) concept (Blanck and Wängberg, 1988). The rational of this concept is that a chronic exposure to a toxicant lead to an increase in the community tolerance that might result from adaptation or acclimatization of populations or from shifts in community composition following the replacement of sensitive species by tolerant species. Community tolerance to a certain toxicant is quantified by measuring responses of physiological endpoints in acute short-term bioassays by comparing the responses of the reference community and the chronically pre-exposed one (Blanck, 2002).

PICT approach has been used on several studies: terrestrial microbes (Díaz-Ravina and Bååth, 1996; Bérard et al., 2014), freshwater biofilms (Tlili et al., 2011a; Bonet et al., 2014), and algal communities (Soldo and Behra 2000; McClellan et al., 2008), but no studies were conducted so far with microbial communities involved in plant litter decomposition. PICT approach has been suggested as a powerful indicator to establish causal relationships between chemicals and their effects on the biota (Tlili et al., 2016b), so using PICT approach associated with the microbial decomposer communities may provide more ecologically relevant information for risk assessment of AgNPs in freshwater environments.

The aim of this study was to determine whether exposure of stream-dwelling microbial decomposers to low concentrations of AgNPs and ionic Ag shifts bacterial and fungal community structure and, thus, increases community tolerance to AgNPs and ionic Ag. To that end, microbial communities associated with leaf litter were exposed to low concentrations of AgNPs and $AgNO_3$, and community tolerance acquisition was measured in short-term bioassays by establishing dose-response curves for several endpoints. We expected that microbial communities pre-exposed to AgNPs and/or $AgNO_3$ would be more tolerant to these toxicants than the control community, probably due to shifts in fungal and bacterial communities and/or to the development of detoxification mechanisms by species within the community. Due to the antimicrobial effect of ionic Ag, we also hypothesized that AgNPs and the ionic metal would have more impacts on bacterial communities, and effects would be more pronounced for the ionic metal.

5.2.Material and methods

5.2.1. Experimental design

Litter bags containing a mix of leaves (oak, alder and poplar leaves) were placed into 0.5 mm mesh bags (16x20 cm) and immersed in a stream located in the Harz mountains, Germany (+51° 42' 0.43", +10° 21' 54.38") to allow microbial colonization. After 7 days, leaf bags were retrieved and transported to the laboratory in a cooling box containing stream water. Colonized leaves were placed in stream water under aeration at 16ºC for two days to stimulate the release of fungal spores and the detachment of bacteria, which were used as inoculum in the microcosm experiments. Dissolved oxygen concentrations and pH of stream water were measured in situ using a Multiline F/set 3 no. 400327 (WTW, Weilheim, Germany) and water samples were collected and transported for quantification of inorganic nutrients (Table 5.1).

Leaves of *Populus* sp. (poplar) were soaked in deionized water and cut into 12 mm diameter discs. The leaf discs were dried at 45ºC for 2 days and then weighed to determine the initial dry mass. After being weighed, leaf discs were leached with Volvic mineral water (Auvergne Regional Park, France; pH=7, Ca=11.5, Cl-=13.5, NO_3=6.3, K=6.2, Na=11.6 mg L^{-1}) and placed in polypropylene microcosms (20x15 cm) with 400 mL of Volvic water (130 leaf discs per microcosm) and 40 mL of the inoculum to ensure microbial colonization of the leaves (ca. 1.8 $x10^5$ spores mg^{-1} leaf dry mass day^{-1}).

After 5 days, the water was renewed and microcosms were exposed to four levels of citrate-coated AgNPs (0, 50, 100 and 200 µg L^{-1}; NanoSys GmbH, Wolfhalden, Switzerland) or one level of ionic Ag concentration (20 µg L^{-1}, added as $AgNO_3$, >99%; Sigma-Aldrich, St. Louis, MO) for 25 days at 16ºC on an orbital shaker at 120 rpm (4 replicates per treatment). Water in the microcosms was renewed every 5 days, and conidial suspensions were preserved in 2% formalin for further fungal identification and counting. After 25 days, leaf discs were sampled to determine Ag accumulation, microbial biomass and diversity, microbial respiration, as well as the activity of extracellular enzyme leucine aminopeptidase (LAP). Leaf discs were also used to test the tolerance acquisition in short-term bioassays to establish dose-response curves for the fungal sporulation, bacterial production, microbial respiration and the activity of LAP.

Table 5.1 - Physical and chemical parameters of the stream water collected at the source of Kleine Lonau stream on Harz Mountains, Germany.

Parameter	Value
pH	7.2
O_2 dissolved (mg L^{-1})	8.8
N-NO_3^- (mg L^{-1})	0.658
N-NO_2^- (mg L^{-1})	0.002
N-NH_4 (mg L^{-1})	0.022
P–PO_4^{3-} (mg L^{-1})	0.005

5.2.2. AgNP characterization

AgNP suspensions of 100 and 200 µg L^{-1} were prepared in fresh and conditioned water retrieved from microcosms for size and surface charge characterization. Conditioned water was obtained from control microcosms, after each water renewal and incubated with AgNPs (50 and 100 µg L^{-1}) every 5 days. The hydrodynamic diameter of AgNPs in the suspensions was measured by dynamic light scattering (DLS), using a Zetasizer (Nano ZS, Malvern Instruments Ltd., Worcestershire, UK), and by nanoparticle tracking analysis (NTA), using a NanoSight LM10 equipped with a LM14 temperature controller (NanoSight Ltd., Wiltshire, UK). The zeta-potential of the AgNPs in the suspensions was measured using the Zetasizer.

5.2.3. Metal analysis

Total Ag concentrations in 50, 100 and 200 µg l^{-1} AgNP suspensions and leaf discs, were determined after acidic digestion with 4 mL 65% HNO_3 in a high-performance microwave digestion unit (MLS-1200 MEGA, Leutkirch, Switzerland) at maximum temperature of 195°C and maximum pressure of 100 bar and before diluting the samples 50 times with nanopure water (1.3% final HNO_3 concentration). Each solution was diluted 50 times with nanopure water, and the total Ag content (isotope ^{109}Ag) was measured by inductively coupled plasma-mass spectrometry, HR-ICP-MS (Element 2 High Resolution Sector Field ICP-MS; Thermo Finnigan, Bremen, Germany). The reliability of the measurements was determined using specific water references (National Water Research Institute, Burlington, Canada). Dissolved Ag from the AgNP suspensions was determined by ultrafiltration for 30 min at 3220 *xg* (Megafuge 1.0R, Thermo Scientific Inc., Waltham, MA) using Ultracel

3k Centrifugal Filter Devices (Amicon Millipore) with a molecular cutoff of 3 kDa (pore size <2 nm). Ag concentration in the filtrate was measured as for the total Ag.

5.2.4. Nutrient quantification

Water samples from each microcosm were analyzed for total phosphorus (TP), soluble reactive phosphorus (SRP), total nitrogen (TN), nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4) and dissolved organic carbon (DOC). Samples were pre-filtered (0.2 µm Nucleopore) and kept at −20°C until analysis. TP was first digested to SRP with $K_2S_2O_8$ (134°C for 30 min) and then determined as PO_4^{3-}. TN was first digested to NO_3^- /NO_2^- with an Oxisolv® (Merck) treatment and allowed to react at 120°C for 45 min. Nitrite and nitrate concentrations in water samples were measured photometrically (FIAstar™ 5010 analyzer, FOSS), according to Wetzel and Likens (1991) and the manufacturer's instructions. DOC was determined as non-purgeable organic carbon (NPOC), where the water samples were treated with 2N HCl. DOC was then analyzed by high temperature combustion in a TOC analyzer (Multi N/C 3100, Analytik Jena AG).

5.2.5. Leaf decomposition

Leaf discs retrieved from each microcosm before and after the experiment were freeze-dried (Christ alpha 2–4; B. Braun, Melsungen, Germany) to constant mass (± 48 h) and weighed to the nearest 0.01 mg.

5.2.6. Fungal sporulation and biomass

Conidial suspensions were mixed with 200 mL of 0.5% Tween 80, filtered (0.45 µm pore size, Millipore, Billerica, MA, U.S.A.), and the retained conidia were stained with 0.05% cotton blue in lactic acid. At least 300 conidia were identified and counted under a light microscope at 400x magnification (Leica Biomed, Heerbrugg, Switzerland).

Fungal biomass on leaves was quantified from ergosterol concentration, according to Gessner (2005). Lipids were extracted from sets of 4 leaf discs by heating (80ºC, 30 min) in 0.8% of KOH/methanol, purified by solid-phase extraction. Ergosterol was quantified by high-performance liquid chromatography (HPLC), using a LiChrospher RP18 column (250 mm x 4 mm, Merck), connected to a liquid chromatographic system (Beckmann Gold System, Brea, CA). The system was run isocratically with HPLC grade methanol at 1.4 mL min^{-1} and 33ºC. Ergosterol was detected at λ=282 nm and its concentration was estimated using standard series of ergosterol (Fluka) in isopropanol.

5.2.7. Bacterial abundance and production

Bacterial abundance was estimated from sets of 3 leaf discs collected before and after the microcosm experiments. Bacteria were detached from leaf discs by sonication at 76 of amplitude for 60 s (samples were cooled on ice after each 20 s of sonication). Bacteria were stained with SYBR Green I diluted 10000x in DMSO (SYBR Green I Nucleids Acid Gel Stain, Lonza, Rockland, ME USA), during 15 min at room temperature in the dark and quantified by flow cytometry. Fluorescent beads (Flowcount flurospheres, Beckman Coulter, Inc. Brea, CA) were added to each sample as an internal standard to normalize cell fluorescence emission and light scatter values. All samples were run on a Gallios flow cytometer (Beckman Coulter) equipped with a laser emitting at 488 nm. The green and red fluorescent signals were collected in the FL1 channel (368 nm) and in the FL3 channel (486 nm), respectively. For each sample run, data for 5,000 events were collected. All data were processed with Kaluza for Gallios software (Partek Inc., St. Louis, MO, USA.), which was also used to separate positive signals from noise.

Bacterial production was estimated using sets of 3 leaf discs per replicate. Leaf discs were put into scintillation vials containing 2.9 ml of filter-sterilized mineral water. Samples were incubated with leucine (final concentration 50 µM: 4.5 µM ^{14}C-leucine + 2.4 M non-radioactive leucine) for 30 min at 15ºC with shaking. Incorporation of leucine was stopped by adding 5% w/v TCA (final concentration), and samples were sonicated for 1 min and filtered on 0.2 µm polycarbonate filter. Both filter and leaf discs were washed twice with 5% TCA, and once with 40 mM leucine, 80% ethanol, and nanopure water. The filter and leaf discs were transferred into a centrifuge tube containing 1.5 mL of an alkaline solution (1.5 M NaOH, 75 mM EDTA, 0.3% SDS) and incubated for 60 min at 90ºC. The samples were cooled down to ambient temperature, centrifuged (10 min at 14000 *xg*) and 250 µL of supernatant transferred to a scintillation vial with 5 mL of Ultima Gold XR scintillation cocktail, and radioactivity was measured in a scintillation analyzer (Tri-Carb 2810 TR, Perkin Elmer).

Leucine incorporated into bacterial protein was expressed in mol g^{-1} litter dry mass day^{-1}, and bacterial production (BP) in g C g^{-1} litter dry mass day^{-1} (Buesing and Gessner, 2003).

5.2.8. Microbial diversity from DNA fingerprinting

DNA was extracted from three leaf discs using an Ultra Clean Soil DNA isolation kit (MoBio Laboratories, Solana Beach, CA), according to the manufacturer instructions. The ITS2 region of fungal rDNA was amplified with the primer pairs ITS3GC and ITS4 and

the V3 region of bacterial 16SrDNA was amplified with the primer pairs 518R and 338F_GC (Duarte et al. 2008). For polymerase chain reaction (PCR) of fungal DNA, 12.5 µL of Go Taq, 0.5 µL of each primer, 1 µL of DNA and 10.5 µL of ultra-pure water were used in a final volume of 25 µL. PCR reagents were purchased from Promega except primers that were from Stabvida. DNA amplification was carried out in a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) (Duarte et al., 2008).

Denaturing gradient gel electrophoresis (DGGE) analysis was performed using a DCodeTM Universal Mutation Detection System (BioRad Laboratories). Samples of 20 µL from the amplification products of 380-400 bp were loaded on 8% (w/v) polyacrylamide gel in 1x Tris-acetate-EDTA (TAE) with a denaturing gradient from 30 to 70% for fungal DNA and 40 to 70 % for bacteria DNA. The gels were run at 55 V, 56ºC for 16 h and stained with Midori Green (Grisp) for 10 min in a shaker at 40 rpm. Gel images were captured under UV light in a ChemiDoc XRS (BioRad).

5.2.9. Microbial respiration

Microbial respiration was measured by using the MicroResp approach (Tlili et al., 2011b), a colorimetric method based on CO_2 production in a closed microplate system. Leaf discs (1 per replicate) were placed in wells of a microplate (Nunc DeepWell, Thermo Scientific Inc.), and 25 mM glucose was added, as a carbon source, to maximize respiration. Microplates were airtight sealed, pairing the wells with a second detection plate that contained a pH indicator embedded in agar with a maximum absorbance at 572 nm. CO_2-trapped absorbance was measured at 572 nm immediately before sealing the well plate, and after 15 h incubation in the dark at 20 ± 1°C. The results were expressed in µg CO_2 mg^{-1} h^{-1}. EC_{50} values were calculated from dose–response curves by plotting microbial CO_2 production at each nano and ionic Ag concentration.

5.2.10. Activity of extracellular enzyme LAP

The activity of leucine aminopeptidase (LAP) was assayed by fluorescent-linked artificial substrate L-leucine-4-methyl-7-coumarinylamide hydrochloride (Leu-AMC [7-amino-4-methylcoumarin], Sigma). LAP cleaves peptides and supplies cells with a nitrogen source. One leaf disc per replicate was incubated at saturating concentrations of the substrate (10 mM) at 15°C in the dark. The reaction was stopped after 1h by adding glycine buffer (1 M, pH 10.4) and the fluorescence was measured at 455 nm upon excitation at 365 nm. The

fluorescence data were converted to concentrations of cleaved substrate analogues based on a calibration curve.

5.2.11. PICT measurements

Induced tolerance of microbial communities to AgNPs and ionic Ag from different treatments was examined via short-term inhibition assays. Leaf discs from each microcosm were exposed for 12 h to increasing concentrations of AgNPs or $AgNO_3$ ranging from 0 to 16500 µg L^{-1} (5 leaf discs per concentration). The inhibition of fungal sporulation, bacterial production, microbial respiration and the activity of LAP were measured as explained previously.

5.2.12. Data analyses

The effects of AgNPs and ionic Ag on leaf mass loss, fungal biomass and sporulation, bacterial production and biomass, activity of LAP and microbial respiration were tested by one-way analyses of variance (one-way ANOVA). Two-way ANOVAs were used to determine the effects of exposure concentrations and the communities exposed to AgNP and $AgNO_3$ on the PICT measurements: bacterial production, fungal sporulation rates, activity of LAP and microbial respiration. Significant differences between control and treatments were analyzed by Bonferroni post-tests (Zar, 2010). No data transformation was done since the data were homoscedastic and had a normal distribution.

Dose-response curves obtained from short-term bioassays were adjusted to four-parameter logistic curves. AgNPs and ionic Ag concentrations leading to 50% effect (EC_{50}) were determined by the nonlinear regression sigmoidal dose–response curves using the Hill slope equation (GraphPad Prism version 6.00 for Windows, San Diego, CA).

DGGE gels were aligned and analyzed with Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analyses of fungal and bacterial community structures were done with PRIMER analytical software (v.6.1.6, PRIMER-E, UK).

5.3. Results

5.3.1. Nanoparticle characterization and chemical analysis

The average particle diameter size measured by DLS in fresh water was 67 ± 3 and 76 ± 1 nm for 100 and 200 µg L^{-1} AgNPs in the suspensions, respectively (Table 5.2). The average size tended to increase in microcosms with leaves conditioned by microorganisms and also with the exposure time. The maximum size observed was 151 ± 7 nm in the water from

conditioned microcosms after 25 days of experiment at the highest AgNP concentration (200 μg L^{-1}). AgNPs in 100 and 200 μg L^{-1} AgNPs in fresh water had a zeta-potential of -16 mV (Table 5.2). More negative charges were measured in water from microcosms with conditioned leaves, and they slightly decreased with the exposure time (Table 5.2). Similar particle sizes of AgNPs in suspensions were determined by NTA (Table 5.2), and AgNP size distributions are shown in Table 5.2.

Table 5.2 - AgNP particle diameter measured by dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) on freshly prepared water (fresh) and water from microcosms with leaves conditioned by microbes (conditioned) (100 and 200 μg L^{-1}). Water in microcosms was renewed every 5 days along 25 days of experiment. The polydispersion index (PdI) and the zeta-potential was measured by Zetasizer. Mean ± SD, n = 3.

Exposure			**DLS**		**NTA**	**Zeta potential (mV)**
AgNP (μg L^{-1})	**Water**	**Time (days)**	**Avg. Diameter (nm)**	**PdI**	**Avg. Diameter (nm)**	
100	Fresh	0	67 ± 3	0.35 ± 0.02	44	-16 ± 2
		5	66 ± 1	0.28 ± 0.03	39	-6 ± 1
100	Conditioned (1st renewal)	0	108 ± 1	0.39 ± 0.02	73	-18 ± 3
		5	107 ± 3	0.52 ± 0.01	69	-15 ± 2
	Conditioned (2nd renewal)	5	100 ± 8	0.36 ± 0.03	69	-16 ± 4
		10	112 ± 6	0.56 ± 0.02	69	-14 ± 3
	Conditioned (3rd renewal)	10	115 ± 9	0.34 ± 0.06	69	-21 ± 3
		15	95 ± 6	0.39 ± 0.06	76	-12 ± 2
	Conditioned (4th renewal)	15	78 ± 9	0.40 ± 0.07	70	-19 ± 1
		20	104 ± 2	0.39 ± 0.03	78	-11 ± 3
	Conditioned (5th renewal)	20	153 ± 5	0.32 ± 0.07	66	-19 ± 1
		25	64 ± 4	0.86 ± 0.15	66	-16 ± 2
200	Fresh	0	76 ± 1	0.44 ± 0.06	45	-16 ± 1
		5	50 ± 2	0.42 ± 0.03	47	-16 ± 1
200	Conditioned (1st renewal)	0	63 ± 1	0.38 ± 0.01	75	-14 ± 1
		5	104 ± 2	0.41 ± 0.04	76	-16 ± 1
	Conditioned (2nd renewal)	5	96 ± 4	0.37 ± 0.07	73	-15 ± 3
		10	108 ± 4	0.61 ± 0.06	74	-14 ± 4
	Conditioned (3rd renewal)	10	77 ± 8	0.41 ± 0.09	48	-17 ± 1
		15	115 ± 5	0.53 ± 0.03	74	-10 ± 2
	Conditioned (4th renewal)	15	101 ± 4	0.44 ± 0.05	65	-21 ± 2
		20	139 ± 7	0.55 ± 0.04	88	-12 ± 2
	Conditioned (5th renewal)	20	77 ± 6	0.43 ± 0.10	69	-18 ± 1
		25	151 ± 7	0.49 ± 0.04	71	-16 ± 1

After 25 days, total Ag concentration in the water with AgNPs was 500 times lower than the concentration added as nanoparticles for all AgNP treatments (Table 5.3). Moreover, the accumulation of Ag on leaves was notable for all treatments, particularly in $AgNO_3$ (12.3 ± 2.9 µg g^{-1} of total Ag) where the concentration was 10 times lower than in the AgNP treatments. The fraction of dissolved Ag in AgNP suspensions was similar across AgNP concentrations and exposure times (< 0.01 µg L^{-1}).

Table 5.3 – Total silver concentration in leaves and in the medium in microcosms contaminated or not with AgNPs and ionic Ag at 16ºC after 25 days. Mean ± SD, n = 4.

	Ag (µg L^{-1})	**Leaves**	**Medium**
		[Ag] (µg g^{-1})	**[Ag] (µg L^{-1})**
Control	**0**	0.10 ± 0.05	0 ± 0.00
AgNPs	**50**	3.19 ± 0.68	0.09 ± 0.04
	100	11.35 ± 2.62	0.16 ± 0.05
	200	14.24 ± 3.38	0.36 ± 0.16
$AgNO_3$	**20**	12.27 ± 2.86	0.06 ± 0.02

A decrease of DOC after 5 days of the experiment was found in all treatments, and ranged from ± 50 to ± 19 mg L^{-1}, while NO_3^- concentration increased along the exposure time, especially for 200 µg L^{-1} AgNPs (from 0.013 to 0.305 mg L^{-1}) and $AgNO_3$ (from 0 to 0.203 mg L^{-1}) treatments (Table S5.1). Oxygen and pH measured in each microcosm showed a slight increase along the exposure time for all treatments (Table S5.1).

5.3.2. Effects of AgNPs and $AgNO_3$ on microbial diversity

In the inoculum used for the microcosm experiments, 15 sporulating fungal species were found on colonizing leaves. After 25 days, 12 sporulating fungal species were found on poplar leaves. The dominant fungal species in the control were *Flagellospora curvula*, followed by *Tetracladium marchalianum* and *Tetrachaetum elegans* (Table 5.4). *Clavariopsis aquatica* mainly occurred in communities exposed to AgNPs and ionic Ag, whereas *Tetrachaetum elegans* became rare.

Table 5.4 - Percentage contribution of each fungal taxon to the total conidial production on leaves in the inoculum and then exposed for 25 days in microcosms to AgNPs (NP1= 50 µg L^{-1}; NP2= 100 µg L^{-1}; NP3= 200 µg L^{-1}) or to $AgNO_3$ (20 µg L^{-1}). -, not detected

Fungal taxa	Inoculum	Control	NP1	NP2	NP3	$AgNO_3$
Alatospora acuminata	0.43	-	0.87	0.70	1.03	0.99
Alatospora pulchella	-	0.26	-	-	-	0.25
Anguillospora crassa	0.36	-	-	-	-	-
Anguillospora filiformis	0.21	0.43	-	-	-	-
Articulospora tetracladia	0.78	2.09	2.83	3.95	4.62	9.16
Clavariopsis aquatica	0.92	-	0.89	3.74	5.26	1.30
Cylindrocarpon sp.	0.14	0.28	0.20	-	0.36	0.24
Dimorphospora foliicola	0.85	-	-	-	-	-
Flagellospora curvula	70.20	80.92	86.53	84.25	77.57	77.12
Fusarium sp.	0.71	-	-	-	-	-
Heliscus lugdunensis	1.14	-	0.28	0.19	0.21	0.23
Lemonniera aquatica	0.28	0.53	0.10	0.30	0.10	0.76
Tetrachaetum elegans	23.47	5.52	1.64	4.49	3.17	2.92
Tetracladium marchalianum	0.36	9.98	6.65	2.40	5.67	7.04
Tricladium chaetocladium	0.14	-	-	-	-	-

Cluster analysis of DNA fingerprints showed that fungal and bacterial communities at the beginning of the experiment were clearly separated from the communities sampled after 25 days (Fig. 5.1a, b). After 25 days, the fungal and bacterial communities exposed to 100 and 200 µg L^{-1} AgNPs and 20 µg L^{-1} $AgNO_3$ grouped together. Fungal communities exposed to 50 µg L^{-1} AgNPs grouped with the controls and were separated from those exposed to $AgNO_3$ (Fig. 5.1b).

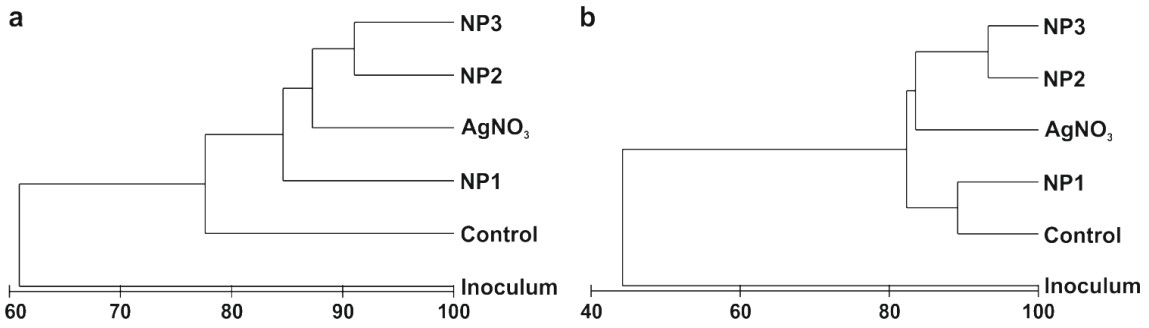

Figure 5.1 - Dendograms from DNA fingerprints based on DGGE of bacterial (a) and fungal (b) communities at the beginning of the experiment (inoculum) and after exposure for 25 days in microcosms to AgNPs (control, NP1= 50 µg L^{-1}, NP2=100 µg L^{-1}, NP3=200 µg L^{-1}) and to $AgNO_3$=20 µg L^{-1}. Clusters were constructed based on UPGMA analyses using the Pearson correlation coefficient.

5.3.3. Effects of AgNPs and $AgNO_3$ on leaf decomposition and microbial functions

After 25 days, leaves lost 60% of their mass. The decomposition of poplar leaves was not significantly affected by the exposure to AgNPs or $AgNO_3$ (one-way ANOVA, $p = 0.0612$) (Fig. 5.2a).

At day 25, fungal sporulation was $1.0x10^5$ spores mg^{-1} leaf dry mass day^{-1} in control microcosms. Exposure to AgNPs had no significant effects on fungal sporulation, while exposure to $AgNO_3$ stimulated sporulation to $2.3x10^5$ spores mg^{-1} leaf dry mass day^{-1} (one-way ANOVA, $p < 0.0001$) (Fig. 5.2b). Fungal biomass on leaves in control microcosms was 371.4 µg g^{-1} leaf dry mass. Only the exposure to 200 µg L^{-1}AgNPs and to $AgNO_3$ increased significantly fungal biomass (one-way ANOVA, $p = 0.0003$) (Fig. 5.2c).

Bacterial production on leaves, was signicantly reduced by exposure to 50 µg L^{-1} AgNPs (one-way ANOVA, $p = 0.0174$) but not to 100 and 200 µg L^{-1} AgNPs or 20 µg L^{-1} $AgNO_3$ (Fig. 5.2d), while a significant decrease in the bacterial abundance was observed upon exposure to 100 µg L^{-1} AgNPs (one-way ANOVA, $p = 0.0007$) (Fig. 5.2e).

LAP activity on leaves was 3.85 nmol mg^{-1} h^{-1} after 25 days in control microcosms and was not significantly affected by exposure to AgNPs or $AgNO_3$ (one-way ANOVA, $p = 0.7599$; Fig. 5.2f). Microbial respiration on leaves was significantly reduced by 100 µg L^{-1} AgNP and $AgNO_3$ when comparing to the control microcosms (one-way ANOVA, $p = 0.0021$), while no effect was observed at 50 and 200 µg L^{-1} AgNPs (Fig. 5.2g).

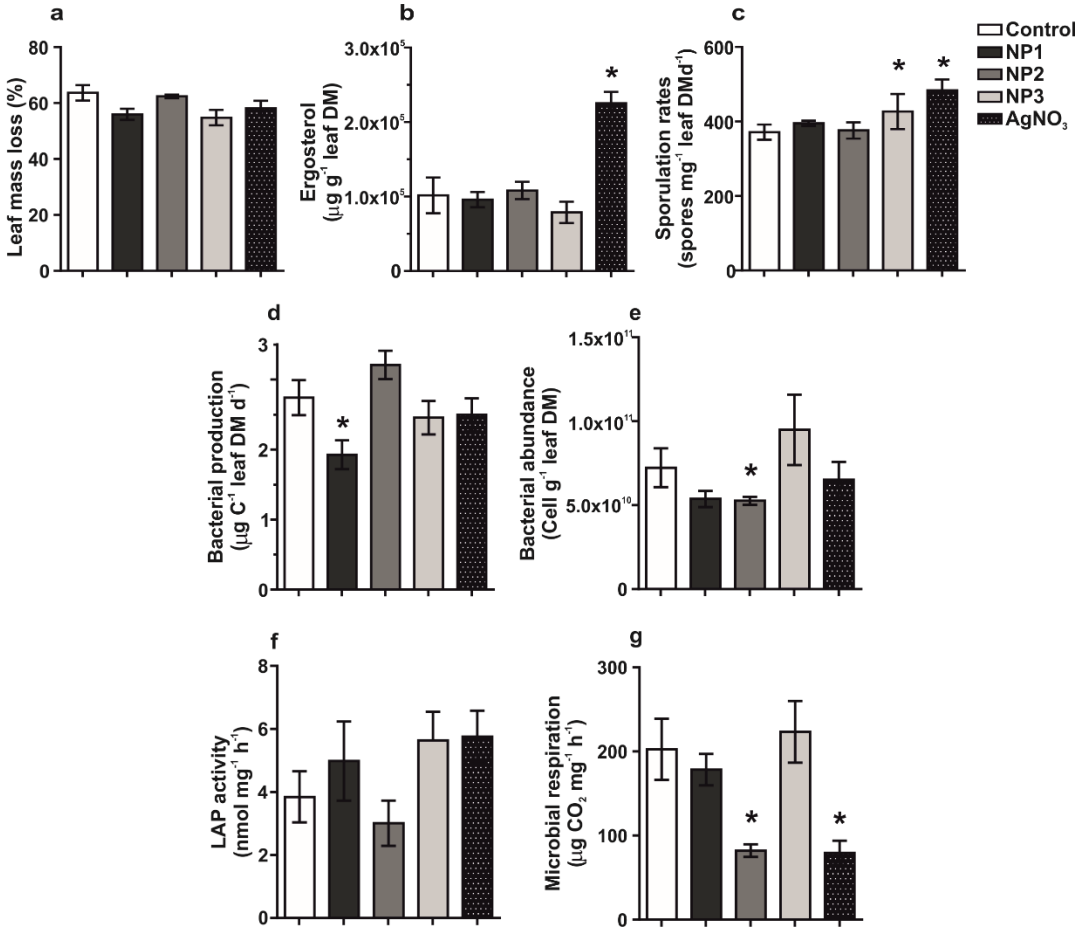

Figure 5.2 - Leaf mass loss (a), fungal sporulation rates (b), ergosterol concentration (c), bacterial production (d), bacterial abundance (e), LAP activity (f) and microbial respiration (g) on colonized leaves that where exposed for 25 days in microcosms to AgNPs (control, NP1= 50 µg L^{-1}, NP2= 100 µg L^{-1}, NP3= 200 µg L^{-1}) and to $AgNO_3$= 20 µg L^{-1}. Asterisks indicate significant differences from the control (one-way ANOVA, $p<0.05$).

5.3.4. PICT measurements

The dose response curves for short-term effects (12 hours) of AgNPs and $AgNO_3$ on fungal sporulation rates, bacterial production, and the activity of LAP in microbial communities exposed to 50, 100 and 200 µg L^{-1} AgNPs and to 20 µg L^{-1} $AgNO_3$ can be found in supplementary material (Fig. 5.3).

Fungal sporulation (Fig. 5.3a, b) and bacterial production (Fig. 5.3c, d) were inhibited by exposure to increasing concentrations of AgNPs and $AgNO_3$ (two-way ANOVA, $p < 0.0001$; Bonferroni tests, $p < 0.05$). For the communities exposed to AgNPs and $AgNO_3$, the concentrations reducing sporulation rate and bacterial production by 50% were higher for AgNPs than for $AgNO_3$ (two-way ANOVA, $p < 0.0001$; Bonferroni tests, $p < 0.05$).

The activity of LAP (Fig. 5.3e, f) and microbial respiration rates were only significantly decreased by exposure to $AgNO_3$ (two-way ANOVA, $p < 0.0001$ and $p = 0.0031$, respectively).

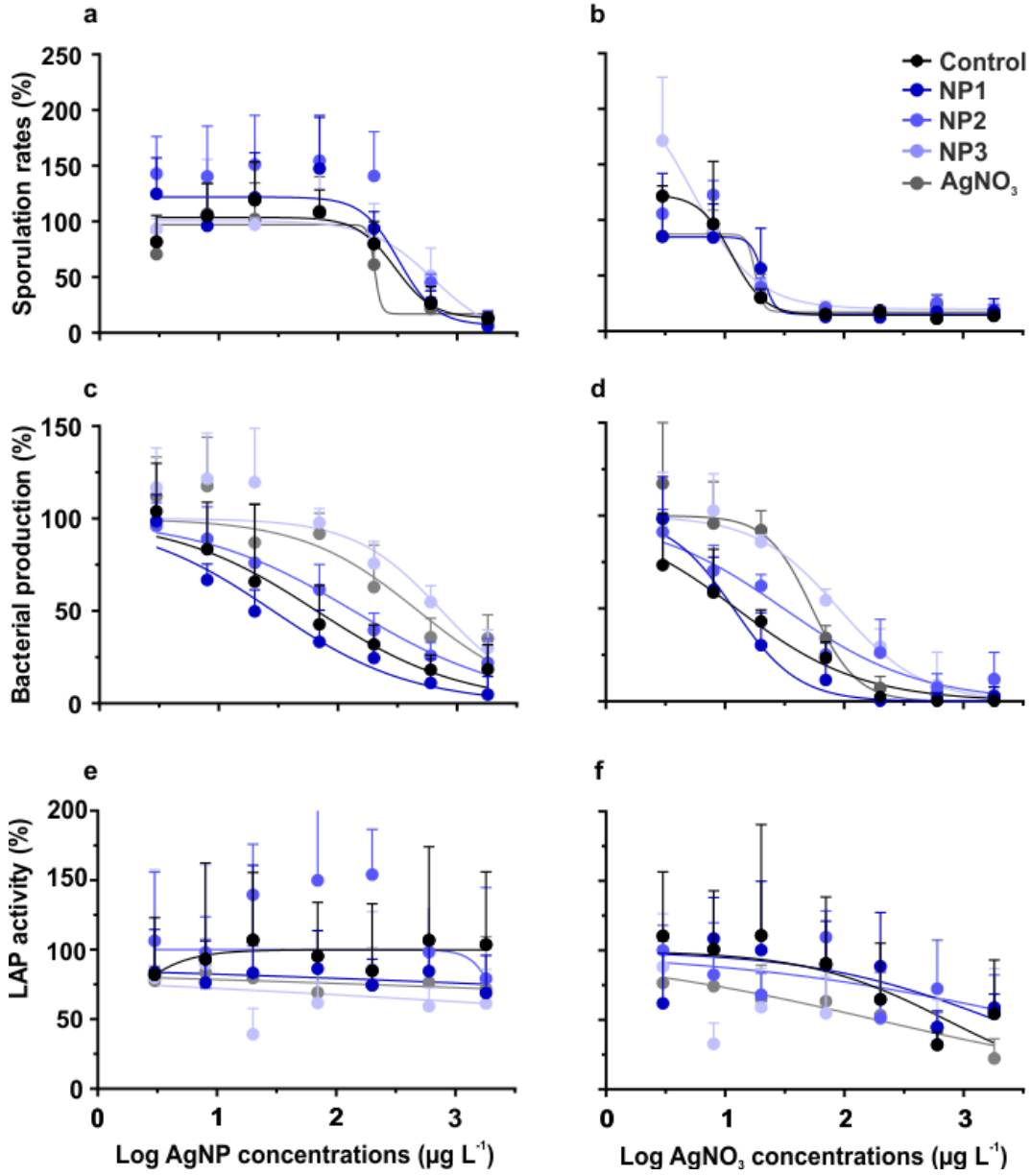

Figure 5.3 - Dose response curves for short-term effects (12 hours) of AgNPs (a, c, e) and $AgNO_3$ (b, d, f) on fungal sporulation (a, b), on bacterial production (c, d), and on the activity of the enzyme LAP (e, f) in microcosms not exposed (Control) or exposed to AgNPs (NP1= 50 µg L^{-1}; NP2= 100 µg L^{-1}; NP3= 200 µg L^{-1}) and to $AgNO_3$ (20 µg L^{-1}).

5.3.5. Tolerance of microbial communities to AgNPs and $AgNO_3$

Based on fungal sporulation, EC_{50} values were not significantly different among treatments in both AgNP and $AgNO_3$ bioassays (Fig. 5.4). On the contrary, EC_{50} values based on

bacterial production were significantly higher for communities exposed to 100 and 200 µg L^{-1} AgNPs and 20 µg L^{-1} $AgNO_3$ than for the control community (Fig. 5.4). Interestingly, the higher the AgNPs concentration to which the communities were exposed, the higher the community tolerance (Fig. 5.4).

It was not possible to calculate the EC_{50} for LAP activity in the AgNP bioassays and for microbial respiration in both AgNP and $AgNO_3$ bioassays because inhibitions of functions did not exceed 50% (Fig. 5.4).

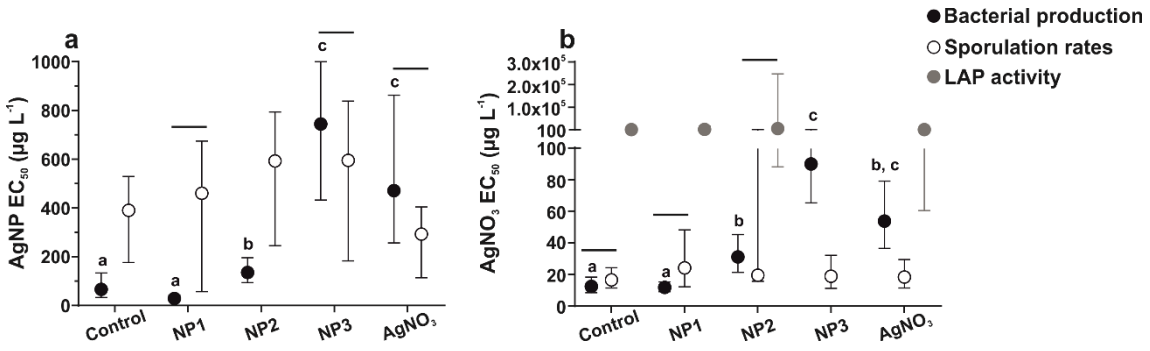

Figure 5.4 - EC_{50} (µg L^{-1}) values for bacterial production, fungal sporulation rates and LAP activity in communities not exposed (control) or exposed to AgNPs (NP1= 50 µg L^{-1}; NP2= 100 µg L^{-1}; NP3= 200 µg L^{-1}) or to $AgNO_3$ (20 µg L^{-1}) in short-term bioassays. C.I. confidence interval (n= 4; α=0.5).

5.4. Discussion

Our study applied PICT concept to plant litter decomposition in freshwater ecosystems, and successfully detected shifts in microbial decomposer communities, namely aquatic fungi and bacteria, caused by AgNPs and $AgNO_3$. Moreover, our results revealed that microbial decomposer communities acquired tolerance to AgNPs and $AgNO_3$ after a chronic exposure to these toxicants.

Microbial endpoints, namely bacterial biomass, were proven an efficient parameter to detect metal-induced tolerance of microbial decomposer communities. Bacterial communities exposed to 100 and 200 µg L^{-1} AgNPs or 20 µg L^{-1} $AgNO_3$ exhibited higher EC_{50} than control communities reflecting community tolerance acquisition. On the other hand, and based on fungal sporulation rates, tolerance was not observed for fungal communities, which may be related to the high variability in the data. Despite the lower EC_{50} values observed for bacterial production than for fungal sporulation rates in control communities, when comparing the communities exposed to higher AgNP and $AgNO_3$

concentrations, it seems that bacteria have the ability to acquire tolerance more efficient than fungi. This makes sense because bacteria have shorter life cycles than fungi, making possible a more effective development of mechanisms of tolerance to Ag at shorter times.

An interesting observation was that processes requiring energy, such as fungal sporulation or the bacterial production (anabolic processes), were more sensitive to AgNPs than LAP activity and microbial respiration rates. The activity of extracellular enzymes and microbial respiration are catabolic processes involved in the breakdown of molecules into smaller units that are either oxidized to release energy or that are used in other anabolic reactions. It is known that the toxicity of AgNPs is related to metabolic activities (Lapresta-Fernández et al., 2012) and also that the tolerance acquisition could result from metabolic processes to cellular detoxification (Tlili and Montuelle, 2011).

For a reliable PICT detection, the choice of specific endpoints is very important to obtain accurate tolerance values (Tlili and Montuelle, 2011, Tlili et al., 2016b). LAP is a protein-degrading extracellular enzyme and is very important for microbial acquisition of nitrogen in aquatic systems (Francoeur and Wetzel, 2003). In our study, short-term exposure to AgNPs did not affect LAP activity, probably because of the presence of metal-resistant species that still have LAP activity at high metal concentrations. Despite the MicroResp technique has been used in several studies (Tlili et al., 2011a; Bérard et al., 2014), we did not find a pattern between communities. Tlili et al. (2011b) stated that some metal salts with nitrogen might interfere with the respiration measurements leading to inaccurate estimates of EC_{50} values. Indeed, we detected an increase in NO_3^- concentration along the exposure time for 200 µg L^{-1} AgNPs and 20 µg L^{-1} $AgNO_3$.

In the case of bacterial community, exposure to 20 µg L^{-1} $AgNO_3$ led to an increased tolerance to AgNPs and vice versa, representing a case of co-tolerance, where a community exposed to a given toxicant becomes tolerant to another one. The occurrence of co-tolerance by metals has been reported previously in other organisms (bacteria: Díaz-Ravina and Bååth, 1996; periphyton: Soldo and Behra, 2000; biofilms: Tlili et al., 2011a). In our case, co-tolerance might be explained by the presence of common mechanisms of detoxification of both nano and ionic forms of Ag and probably related to the dissolution of Ag^+. The toxicity mechanisms of AgNPs are still unclear, but a great part can be explained by the effects of dissolved Ag^+ and other dissolved silver species derived from AgNPs, such as $AgCl^-$ (aq) and AgOH (aq) (Marambio-Jones and Hoek, 2010; Levard et al., 2012; Yu et al., 2013). The presence of halides such as Cl^- or Br^- can decrease silver

bioavailability in the media and increase silver resistance in bacteria, as observed for *Escherichia coli* (Gupta et al., 1998).

In our study, some sporulating fungal species were only found in communities exposed to AgNPs or $AgNO_3$. This could be due to the development of tolerance to AgNPs: the inhibition of sensitive species would be compensated by an increased importance of tolerant species as suggested by the PICT concept (Blanck et al., 1988). DNA fingerprints revealed shifts in the structure of bacterial and fungal communities after 25 days of exposure. This agrees with results obtained in a microcosm study with algal communities, in which algal community composition changed after exposure to metal concentrations that induced an increase in the community tolerance (Soldo and Behra, 2000). Also, Pradhan et al. (2011) demonstrated shifts in the structure of fungal and bacterial communities based on DNA fingerprints caused by the exposure of microbial decomposers to CuONPs and AgNPs.

It is very interesting that the chronic exposure to AgNPs and $AgNO_3$ affected the functional endpoints in our study in distinct ways. AgNPs did not affect leaf mass loss or fungal sporulation rates after 25 days of exposure, even though fungal sporulation is severely inhibited by ionic (Funck et al., 2013; Tlili et al., 2016a) and nano Ag (Pradhan et al., 2011). On the contrary, $AgNO_3$ stimulated fungal sporulation and biomass, which can be explained by nutrient enrichment, since the ionic Ag was added as $AgNO_3$. Increased concentrations of nitrogen have been reported to stimulate both fungal and bacterial activities on decomposing leaves (Sridhar and Bärlocher, 2000; Ferreira, Gulis and Graça, 2006; Fernandes et al., 2014). Another explanation relies on the fact that at low concentrations, metals can stimulate the reproduction and growth of several aquatic organisms (Calabrese and Blain, 2005; Batista et al., 2012).

The scientific community defend that assessing the potential harm of AgNPs in aquatic environments requires a good characterization of AgNPs (Fabrega et al., 2011) and AgNP properties should ideally be quantified in real exposure situations, taking into consideration the exposure periods and the medium used in the ecotoxicological studies (Domingos et al., 2009; Holden et al., 2016). In our study, AgNP characterization was performed using DLS and NTA and every 5 days of exposure in fresh and conditioned water to check interactions between AgNPs, and AgNPs and biomolecules released by the metabolism of microorganisms, as well as by particulate organic matter resulting from microbial activity. An increase in the average particle size diameter was observed, especially with exposure time in water from microcosms with leaves conditioned by microbes, probably due to the

accumulation of natural organic matter along time. NP associations with natural organic matter are known as strongly affect the NP surface chemistry and bioaccumulation (Fabrega et al., 2009; Pradhan et al., 2015b). Despite the slight increase, the AgNPs were still in a small average size and we can considerer that NPs were stable, since no strong agglomeration was observed as confirmed by the polydispersity index.

In our study, $AgNO_3$ inhibited microbial communities more than AgNPs for all parameters analyzed, even using lower concentrations than those of the nano metal. These findings suggest that the $AgNO_3$ might be more toxic than the AgNPs, as reported in other studies testing short-term (algae: Navarro et al., 2008b; periphyton: Gill-Allué et al., 2015) and chronic effects (microbial decomposers: Pradhan et al., 2011). Aquatic fungi can growth within decomposing leaf tissues, and this may constitute an effective protection against NPs than from ions. Indeed, a higher percentage of Ag ions was found on leaves exposed to $AgNO_3$ than to AgNPs.

In our study, PICT revealed to be a successfully ecotoxicological tool by combining structural and functional measurements at the community level. The impact of AgNPs on the structure and functions of microbial communities involved in leaf litter decomposition can have profound effects on aquatic ecosystem functioning (Fabrega et al., 2011; Pradhan et al., 2011; Tlili et al., 2016a), so studies combining metal NPs with community approaches, like PICT, may provide a better understanding of mechanisms of toxicity triggered by NPs, helping to assess potential impacts of AgNPs on aquatic ecosystems.

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Chapter 6

Impacts of AgNPs on shredder feeding behaviour and detrital processing varies with the exposure route

Abstract

The release of silver nanoparticles (AgNPs) into freshwater ecosystems has increased over the last decade as their commercial use has expanded. The AgNPs and the ionic Ag released from the NPs can have toxic effects on aquatic species and may compromise important ecological processes, such as organic matter decomposition. However, little is known about how species interactions in multi-trophic systems will affect such ecosystem process when exposure routes of AgNPs vary. The goal of this study was to assess the effects of environmentally realistic concentrations of AgNPs and Ag^+ on trophic interactions between aquatic invertebrates and associated ecosystem process. Specifically, we evaluated the importance of both direct (via water) and indirect (via diet) exposure routes by exposing a simplified detrital food web, comprising leaf litter, microbes, a shredder species and collector species to: (i) water contaminated with AgNPs (0, 1, 100 µg L^{-1}) or $AgNO_3$ (0, 0.1 µg L^{-1}), and (ii) leaves contaminated for 6 days with AgNPs and $AgNO_3$ at the same concentrations. Shredders (*Gammarus pulex*, Gammaridae, Amphipoda) and collectors (*Habrophlebiodes sp.*, Leptophlebiidae, Ephemeroptera) were placed in laboratory feeding containers where collectors had access to fine particulate organic matter (FPOM) produced by *G. pulex*, but were separated by a mesh screen. Leaf consumption and FPOM production by *G. pulex*, fungal biomass and decomposition activity were determined after 15 days. The stress induced by AgNPs and Ag^+ in both invertebrate species was assessed by measuring the activity of antioxidant and neuronal enzymes. We found that microbial decomposition was lower by direct exposure to AgNPs and Ag^+, whereas leaf consumption by *G. pulex* only decreased when leaves were contaminated with the lowest concentration of AgNPs. There were no effects on FPOM production in both exposure routes. Changes in the activity of the key antioxidant enzyme catalase indicated that both shredders and collectors were under stress caused by AgNPs and Ag^+, mainly in response to direct exposure. Overall, our results demonstrated that ecological effects on different functional groups of stream invertebrates vary with exposure route to AgNPs at environmentally-realistic concentrations. Thus, the route by which stream biota is exposed to AgNPs will influence the impacts of AgNPs on ecosystem processes.

6.1.Introduction

In freshwater ecosystems, plant litter decomposition is a key process driven by a range of interacting organisms that form a detrital processing chain (Gessner et al., 2007). Within this chain, microbial decomposers, such as fungi (aquatic hyphomycetes), play a major role in leaf litter decomposition (Baldy et al., 2002; Pascoal and Cássio, 2004) and enhance nutritional value of leaves to shredder consumption (Graça, 2001). Microbial decomposers and shredders transform this coarse particulate organic matter (CPOM) into fine particulate organic matter (FPOM), which consist of small particles resulting directly from litter fragmentation, spores released by fungi, and faeces produced by invertebrates (Allan and Castillo, 2007). These materials are food resources for filter feeders and collector-gatherers (Cummin and Klug, 1979). By this route, the shredders can promote growth or increase survival of the collector organisms within these detrital processing chains (Campus et al., 2014). The interactions among microbes, shredders and collectors mean that detrital processing may be affected by stressors acting on any group. Microorganisms and invertebrates respond differently to stress, being sensitive to various types of pollutants (Wallace et al., 1996; Duarte et al., 2004; Batista et al., 2012; Pradhan et al., 2012, 2015). A major limitation in understanding the effects of stressors on ecosystems is that most of the studies focus on single species, excluding the possible additive or interactive effects when multi-trophic systems are considered. Experimental designs considering multi-trophic interactions will help to predict potential effects among trophic levels in naturally-occurring complex food webs (Englert et al., 2012; Kalcíková et al., 2014).

Nanoparticles (NPs) are emerging pollutants that may affect detrital processing chains. Due to their antimicrobial properties, silver nanoparticles (AgNP) are among the most commonly produced NPs (The Project on Emerging Nanotechnology, 2013), and are found in products such as cosmetics, textiles, food packaging and disinfection products. The growing production and use of AgNPs has resulted in their release into freshwater ecosystems, where the nano or/and the ionic form can have toxic effects on aquatic species (Moore, 2006; Navarro et al., 2008a; Fabrega et al., 2011) and compromise important ecological processes such as organic matter decomposition (Pradhan et al., 2011; Tlili et al., 2016). The mechanisms of AgNPs toxicity are poorly understood (Völker et al., 2013) but it is endorsed that AgNP toxicity is mainly caused by the dissolved Ag ions from the NPs (Navarro et al., 2008b; Wang et al., 2012). Previous studies suggest the ionic metals are toxic to components of the detrital processing chain. For example, Batista et al. (2012)

showed that exposure to cadmium strongly decreased leaf decomposition by microbes and also inhibited the leaf consumption by invertebrate shredders.

The mechanism underlying the effects of NPs on leaf decomposition is not well understood because experiments are rarely conducted under environmentally-relevant conditions, i.e. at low concentrations in multi-trophic communities. It is difficult to estimate the concentrations of AgNPs released to the environment at any given time, but predicted environmental concentrations (PECs) for AgNPs in surface waters ranged from 0.088 to 10 000 ng L^{-1}, representing a factor of 10^5 (Gottschalk et al., 2013; Mueller and Nowack, 2008). Previous ecotoxicological studies of AgNPs were conducted at high exposure concentrations (Pradhan et al., 2011) that are probably unrealistic in an environmental context. Despite the challenges of working with environmentally-relevant concentrations, it has been shown that sub-lethal impacts of NPs are better indicators of physiological changes that are important for ecosystem processes (feeding activity: Pradhan et al., 2012, 2015; oxidative stress: Buffet et al., 2011; Mouneyrac et al., 2014). These impacts may have important ecological consequences through the exposure routes and mechanisms of AgNPs other than the direct exposure through water.

Research on the effects of AgNPs on aquatic invertebrates have focussed predominantly on assessing toxicity of ionic and nano Ag on aquatic invertebrates through waterborne exposures (Zhao and Wang, 2012; Blinova et al., 2013; Ali et al., 2014), with less attention paid to dietary exposure (Croteau et al., 2011; Zhao and Wang, 2011; Mouneyrac et al., 2014). Despite several studies proved that food is an important uptake source for contaminants in aquatic organisms (Wang, 2002; Wilding and Maltby, 2006; Quintaneiro et al., 2014), environmental regulations do not take into account the potential impact of contaminated food as a source of metals or NPs to aquatic organisms (Brinkman and Johnston, 2008). In freshwater ecosystems, the release of AgNPs is likely to persist and bioaccumulate either by aqueous uptake or dietary exposure (Frabega et al., 2011; Bundschuh et al., 2016). For example, NPs may adhere to the cell walls of algae, which in turn may be ingested by filter-feeders, thus transferring toxicants to higher trophic levels (Baun et al., 2008). Dietary exposure to NPs can lead to higher total body concentrations in predatory organisms when they consume preys that have accumulated NPs. Recently, Chae and An (2016) demonstrated that silver nanowires are transferred through food chains compromising algae, water fleas and fish, and eventually might be accumulated in organisms at higher trophic levels, potentially including humans. Limited information exists on the potential bioaccumulation and trophic transfer of AgNPs in aquatic organisms,

limiting our ability to predict the impacts of AgNPs on community composition and ecosystem functioning (Rohr et al., 2006; Wang, 2013).

We sought to better understand how species interactions in multi-trophic systems would affect ecosystem processes under various exposure routes of AgNPs and Ag^+ at environmentally-realistic concentrations. Specifically, we assessed both direct (via water) and indirect (via diet) exposure routes by exposing a simplified detrital food web, comprising leaf litter, microbes, a shredder species (*Gammarus pulex*, Gammaridae, Amphipoda) and collector species (*Habrophlebiodes* sp., Leptophlebiidae, Ephemeroptera) to AgNPs or $AgNO_3$. We tested whether: (1) exposure to AgNPs or Ag^+ would affect the microbial communities colonizing leaf litter; (2) the exposure route would influence the shredder feeding behaviour; and (3) exposure to AgNPs or Ag^+ would alter physiological responses of the collector inducing stress through waterborne exposure (direct) or shredder-mediated route (i.e. via FPOM).

We hypothesised that even at environmentally-realistic concentrations of AgNPs, the different organisms would be affected, especially the shredders, since they have been considered among the most sensitive aquatic invertebrates to various chemicals (Alonso and De Lange, 2010; Canivet and Gilbert, 2002). Moreover, the effects of AgNPs would be more pronounced via dietary exposure, because invertebrate shredders prefer to feed on plant litter colonized by microbes, predominantly fungi, whose activities increase the plant litter palatability (Graça, 2001). Finally, we expected that the animals would accumulate silver in their bodies, especially by dietary exposure.

6.2.Materials and methods

6.2.1. Microbial colonization of leaves

Oak leaves (*Quercus robur* (L.)) were collected immediately before abscission and dried at room temperature. In October 2014, sets of oak leaves were placed into 0.5 mm mesh bags (16x20 cm) and bags were immersed at different streams located in the Harz Mountains (Germany) to allow microbial colonization. Dissolved oxygen and pH were measured in situ using a Multiline F/set 3 no. 400327 (WTW). Stream water samples were collected in plastic containers and transported in a cool box (4 ºC) for quantification of inorganic nutrient concentrations (Table S6.1).

After 4 weeks, leaf bags were retrieved and transported to the laboratory, where leaves were soaked in deionized water and cut into 12 mm diameter disks.

6.2.2. Collection of invertebrates and acclimation to the laboratory

Shredders (*Gammarus pulex*, Gammaridae, Amphipoda) and collectors (*Habrophlebiodes* sp., Leptophlebiidae, Ephemeroptera) were collected at an unpolluted site of a lowland stream in Germany (53.1°N, 13.1°E) and transported to the laboratory in plastic containers with stream water and sand. Mayflies and gammarids were chosen to represent collector and shredder feeding groups, respectively. These species were selected because they are widely distributed (Culver et al., 1995), and gammarids are considered to contribute largely to leaf litter breakdown (Dangles et al., 2004), and mayflies contribute to the energy transfer as they participate in FPOM processing during leaf litter decomposition (Cummin and Klug, 1979).

In the laboratory, animals were placed in an aquarium with filtered (MN GF-3 filter paper, Macherey–Nagel, Germany) and sterile mineral water and sand under aeration, at 15ºC with a 12 h light: 12 h dark photoperiod, and were allowed to feed on leaves.

6.2.3. Acute lethality tests

To establish a range of sublethal concentrations to be used in the feeding experiments, acute lethality tests were performed to evaluate the sensitivity of the shredders and collectors to AgNPs. Invertebrate shredders and collectors were starved for 24 h and placed in plastic containers with 20 mL of AgNPs suspensions (n=8). The animals were exposed to 7 levels (0.1 to 1000 µg L^{-1}) of AgNPs prepared in sterilized mineral water. The animals were incubated for 96 h at 15ºC, under a 12 h light: 12 h dark photoperiod. The animals were not fed during the exposure period. In each 24 h, the animals that did not show any movement after mechanical stimulation were considered dead and mortality was recorded.

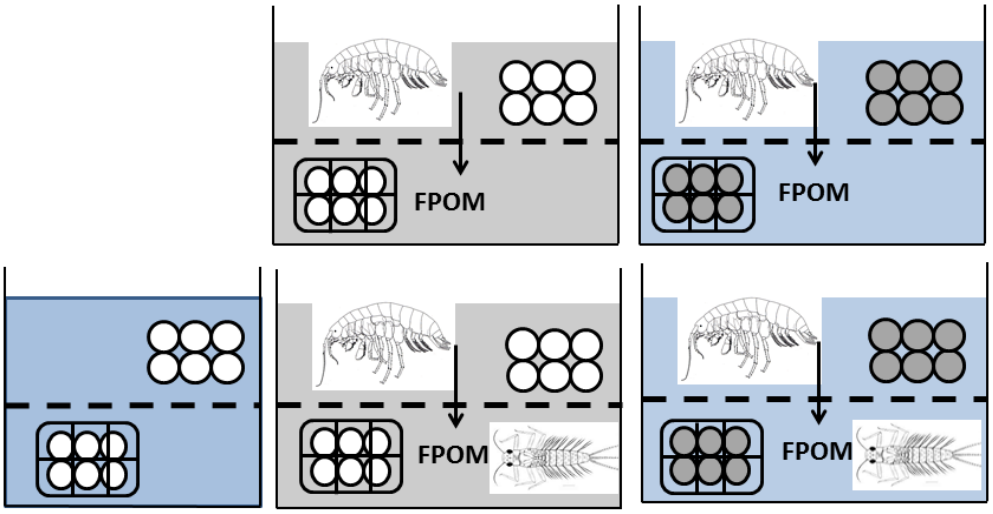
6.2.4. Invertebrate feeding experiment

Shredders (one animal) and collectors (two animals) were placed in laboratory containers (Fig. 6.1). The containers had two chambers separated by a 0.5 mm mesh; an upper chamber containing the shredder and six leaf disks, and a lower chamber containing fine gravel and the collectors (Fig. 6.1). This system allowed collectors to access to fine particulate organic matter (FPOM) produced by *G. pulex*. The microcosms were filled with 400 mL mineral water (Auvergne Regional Park, France; pH=7, Ca=11.5, Cl^-=13.5, NO_3=6.3, K=6.2, Na=11.6 mg L^{-1}). Silver was added to the stream water as citrate coated AgNPs (NanoSys GmbH, Wolfhalden, Switzerland) or $AgNO_3$ (>99%, Sigma-Aldrich, St. Louis, MO). The concentrations used were based on the 96 h LC_{20} values obtained for shredders and

collectors in the acute lethally tests (100 µg L^{-1}). Moreover, we tested one more AgNP concentration 100x lower than the LC_{20} (1 µg L^{-1}).

To assess the direct effects via contaminated water, microcosms with mineral water were supplemented with AgNPs (1, 100 µg L^{-1}) and $AgNO_3$ (0.1 µg L^{-1}) and six microbially-colonized leaf disks not previously exposed to AgNPs or $AgNO_3$. To test the indirect effects via food, microcosms were supplemented with mineral water (without AgNPs or $AgNO_3$) and six microbially-colonized leaf disks that were contaminated for 6 days with AgNPs and $AgNO_3$ at the same concentrations mentioned above. Additional microcosms with mineral water and microbially-colonized leaf disks unexposed to nano and ionic Ag served as controls. Duplicates for all the treatments were done without the mayfly for FPOM control. A total of 140 containers were used (10 replicates).

For determining the contribution of microorganisms to leaf litter decomposition, an equal number of contaminated or uncontaminated leaf disks to AgNPs and $AgNO_3$ was enclosed in 0.5 mm fine mesh bag (to prevent the access of invertebrates) and placed in each replicate microcosm. All microcosms were aerated with air pumps and incubated for 15 days at 15ºC under a 12 h light: 12 h dark photoperiod. Solutions were renewed by 50 % every 5 days and survivorship was registered twice a day during the experiment. At the end of the experiment, leaf disks were frozen and lyophilized to a constant weight; and animals were kept at -80ºC.



Treatment	Control	Via water exposure	Via dietary exposure
Medium	Mineral water	1 µg L^{-1} AgNP 100 µg L^{-1} AgNP 0.1 µg L^{-1} Ag^{+}	Mineral water
Oak leaf disk	Mineral water	Mineral water	1 µg L^{-1} AgNP 100 µg L^{-1} AgNP 0.1 µg L^{-1} Ag^{+}

Figure 6.1 – Experimental setup for the invertebrate feeding experiment. Shredders and collectors were placed in laboratory containers with two chambers separated by a 0.5 mm mesh; an upper chamber containing the shredder and oak leaf disks, and a lower chamber containing collectors and oak leaf disks enclosed in 0.5 mm fine mesh bag (to assess microbial decomposition). The animals were exposed via water or via food (oak leaves) to AgNP and Ag^{+}.

6.2.5. Chemical analyses

6.2.5.1. AgNP characterization and metal analysis

AgNP suspensions of 100 µg L^{-1} were prepared in fresh and conditioned water retrieved from microcosms for size and surface charge characterization. Conditioned water was obtained from control microcosms, after each water renewal and incubated with AgNPs (100 µg L^{-1}) every 5 days. The hydrodynamic size, dispersity and the surface charge of AgNPs in the suspensions were examined by dynamic light scattering (DLS) and zeta potential using a Zetasizer (Nano ZS, Malvern Instruments Ltd., Worcestershire, UK).

The total Ag concentration (isotope ^{109}Ag) in the leaves and in the invertebrate shredders was determined after acid digestion, by high resolution inductively coupled plasma-mass

spectrometry, HR-ICP-MS (Element 2 High Resolution Sector Field ICP-MS; Thermo Finnigan, Bremen, Germany).

6.2.5.2. Nutrient analysis

Water samples from each microcosm were analyzed for total phosphorus (TP), soluble reactive phosphorus (SRP), total nitrogen (TN), nitrite (NO_3^-), ammonia (NH_4) and dissolved organic carbon (DOC). Samples were pre-filtered (0.2 μm Nucleopore) and kept at −20°C until analysis. TP was first digested to SRP with $K_2S_2O_8$ (134°C for 30 min) and then determined as PO_4^{3-}. TN was first digested to NO_3^-/NO_2^- with an Oxisolv® (Merck) treatment and allowed to react at 120°C for 45 min. Nitrite concentrations in water samples were measured photometrically (FIAstar™ 5010 analyzer, FOSS), according to Wetzel and Likens (1991) and the manufacturer's instructions. DOC was determined as nonpurgeable organic carbon (NPOC), after the water samples being treated with 2N HCl. DOC was then analyzed by high temperature combustion in a TOC analyzer (Multi N/C 3100, Analytik Jena AG).

6.2.6. Leaf decomposition and fungal biomass

Leaf disks retrieved from each microcosm were freeze-dried (Christ alpha 2–4; B. Braun, Melsungen, Germany) to constant mass (± 48 h) and weighed to the nearest 0.01 mg.

Fungal biomass on leaves was quantified from ergosterol concentration, according to Gessner (2005). Lipids were extracted from sets of 4 leaf disks by heating (80 ºC, 30 min) in 0.8% of KOH/methanol, purified by solid-phase extraction. Ergosterol was quantified by high-performance liquid chromatography (HPLC), using a LiChrospher RP18 column (250 mm x 4 mm, Merck), connected to a liquid chromatographic system (Darmstadt, Germany; HPLC UltiMate 3000 LC Systems, Thermo Scientific, CA, USA). The system was run isocratically with HPLC grade methanol at 1.4 mL min^{-1} and 33ºC. Ergosterol was detected at λ=282 nm and its concentration was estimated using standard series of ergosterol (Fluka) in isopropanol.

6.2.7. Leaf consumption rates and FPOM production by the shredder G. pulex

The dry mass (DM, mg) of leaves consumed by the invertebrate (L_e) was determined as (L_i - L_f) - (L_i × (C_i - C_f)/C_i), where L_i and L_f are the initial and final dry mass (mg) of leaves exposed to the invertebrates, respectively, and C_i and C_f are the initial and final dry mass (mg) of control leaves (inaccessible to invertebrate), respectively.

Leaf consumption rate by the invertebrate was calculated as $L_e/(I_f \times t)$, where If is the invertebrate dry mass (mg) at time t (day 15), and results were expressed as mg leaf DM mg $^{-1}$ animal DM d^{-1} (Ferreira et al., 2010). Total consumption rate was determined as $((C_i - C_f) + L_e)/t$ and expressed as g leaf DM g^{-1} d^{-1}.

After the feeding experiments the fine particulate organic matter (FPOM), i.e., invertebrate feces and leaf detritus, was collected and filtered on membranes (GF-75, glass microfiber filters 0.45 μm pore size, Whatman, Buckinghamshire, UK). FPOM on the membranes was dried at 60ºC for ± 48 h before being weighed to the nearest 0.001 mg.

6.2.8. Activity of antioxidant and neuronal enzymes

The activity of three antioxidant enzymes, glutathione S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD), and the neuronal enzyme, cholinesterases (ChEs) were measured in the invertebrate shredder *G. pulex* and in the collector *Habrophlebiodes* sp. Pools of two animals per treatment (3 replicates) were homogenized (Ultraturrax IKA, Staufen, Germany) in 1:10 (w/v) 100mM phosphate buffer (containing 2mM EDTA; pH 7.8), plus 100 mM solution of phenylmethylsulfonyl fluoride (PMSF). The homogenates were centrifuged (10,000 ×g for 20 min, at 4°C) to separate the postmitochondrial supernatant (PMS). The PMS from body or head tissues was divided into aliquots and stored at -80°C for protein quantification and evaluation of the activities of stress responsive enzymes.

Catalase activity was measured spectrophotometrically as the decrease in absorbance at 240 nm (ε = 43.6 $M^{-1}cm^{-1}$) due to dismutation of hydrogen peroxide (H_2O_2) according to Beers and Sizer (1951). GST activity was determined spectrophotometrically at 340 nm (ε = 9.6 mM cm^{-1}) by monitoring the formation of 1-glutathion- 2,4-dinitrobenzene, resulting from the conjugation of the substrate, 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione reduced form (GSH), as described by Habig et al. (1974). Cholinesterases (ChEs) activity was determined by measuring the rate of production of thiocholine as acetylthiocholine is hydrolysed at λ=414 nm in a spectrophotometer (ε = 13.6 mM cm^{-1}). SOD activity was quantified following the protocol described in the superoxide dismutase assay (RANSOD, Randox, UK).

All enzymatic activities were calculated in nmoles min^{-1} mg^{-1} protein, except SOD which was expressed as SOD Unit mg^{-1} protein. Protein content was quantified according to Bradford (1976) using BSA as standard. Protein concentration in PMS from the animals was quantified according to Bradford (1976) in a 96-well flat bottom microplate, using

bovine serum Albumin (BSA) as standard. Calibration curves were constructed with 0.1, 0.2, 0.5 and 1 mg mL^{-1} and the protein concentration was expressed per dry mass (DM) of animal.

6.2.9. Data analyses

In the acute toxicity tests, mortality of the invertebrate shredders and collectors was recorded, and the concentration inducing 20% of death (LC_{20}) at 96 h of exposure with the respective 95% C.I. was calculated using PriProbit 1.63 (Sakuma, 1998; http://bru.gmprc.ksu.edu/proj/priprobit/download.asp). Repeated-measures analysis of variance (ANOVA) was used to test the effects of concentrations of AgNPs on the percentage of animal survival in the acute lethality test with matched observations of exposure time (Zar, 2009).

For response variables in the main experiment (leaf mass loss, fungal biomass, leaf consumption and FPOM production by the shredders and activity of enzymes from shredders and collectors) we tested whether the effect of each treatment (i.e. 6 levels; water and dietary exposure of each AgNPs and $AgNO_3$ concentrations) was different from those of control microcosms using multiple comparisons following a linear mixed model with the presence or absence of mayflies as a random effect. The effect of treatment on mayfly enzymes was analysed with a single-factor analysis of variance, followed by post-hoc t-tests.

To identify the interactive effects of exposure route and silver concentration, we used multiple-factor ANOVAs where the response was expressed relative to the control. The interactive effect of concentration (0.1 μg L^{-1} $AgNO_3$, 1 μg L^{-1} NP and 100 μg L^{-1} NP), exposure route (2 levels; water and dietary) and mayflies (2 levels; present or absence) was analysed with a three-factor ANOVA.

All analyses were conducted in R program (R Core Team 2013) using the lme function (nlme package; Pinheiro et al., 2015) for mixed models.

6.3.Results

6.3.1. Nanoparticle characterization and chemical analysis

The average hydrodynamic diameter size of AgNPs (100 μg L^{-1}) measured by DLS in fresh water was 67 ± 3 nm (Table 6.1). This average size of NPs tended to increase in microcosms with leaves conditioned by microorganisms and with the exposure time. After 15 days of the experiment, AgNP size was 93 ± 30 nm. The AgNPs (100 μg L^{-1}) in fresh water had a

zeta-potential of -16 ± 2 at the beginning of the experiment (Table 6.1). The negative charges of AgNPs were relatively higher in microcosms with leaves conditioned by microorganisms, and did not show any considerable variation with exposure time (-19 ± 2 mV after 15 days). The polydispersity index (PdI) showed more stable AgNPs when prepared in fresh water than in conditioned water, indicating monodispersed suspensions at the beginning of the experiment. The PdI had a tendency to increase in microcosms with leaves conditioned by microorganisms and also with the exposure time (Table 6.1).

Table 6.1 - AgNP particle diameter measured by dynamic light scattering (DLS) on freshly prepared water (fresh) and water from microcosms with leaves conditioned by microbes (conditioned) (100 µg L^{-1}). Water in microcosms was renewed every 5 days along 15 days of experiment. The polydispersion index (PdI) and the zeta-potential was measured by Zetasizer. Mean ± SD, n = 3.

Sample		**DLS**			**Zeta potential (mV)**
Concentration (µg L^{-1})	**Water**	**Exposure time (days)**	**Avg. Diameter (nm)**	**PdI**	
100	Fresh	0	67 ± 3	0.35 ± 0.02	-16 ± 2
		5	66 ± 1	0.28 ± 0.03	-6 ± 1
100	Conditioned (1^{st} renewal)	0	68 ± 20	0.45 ± 0.15	-18 ± 3
		5	88 ± 30	0.69 ± 0.06	-17 ± 3
	Conditioned (2^{nd} renewal)	5	89 ± 19	0.54 ± 0.03	-17 ± 2
		10	100 ± 30	0.82 ± 0.13	-16 ± 2
	Conditioned (3^{rd} renewal)	10	85 ± 30	0.58 ± 0.09	-19 ± 2
		15	93 ± 30	0.58 ± 0.25	-19 ± 2

After 15 days, the concentration of Ag in leaves and in the shredders bodies consistently increased with increasing concentrations of AgNPs (Table 6.2). The Ag accumulation on the leaves in the treatments were the water was contaminated with AgNPs was high, especially in the 100 µg L^{-1} treatment (160.58 ± 44.00 ng g^{-1}, Table 6.2). Ag accumulation in the shredders followed the same pattern than the Ag accumulation on leaves, especially in treatments where water was contaminated with 100 µg L^{-1} AgNPs (29.34 ± 16.7 ng g^{-1}).

Moreover, the total Ag found on the leaves and in the shredder exposed to Ag^+ was notable, even because the Ag concentration was 10× lower than that used in the AgNP treatments (Table 6.2).

Table 6.2 - Total silver concentration (ng g^{-1}) in the leaves and in the invertebrate shredders exposed via water and via dietary (oak leaves) to AgNPs and Ag^+ during 15 days. Mean ± SD, n = 3.

Treatment	Ag (µg L^{-1})	Exposure route	Leaves	Gammarus
			[Ag] (ng g^{-1})	[Ag] (ng g-1)
Control	**0**	-	0.32 ± 0.21	0.20 ± 0.53
AgNP	**1**	Water	1.79 ±1.27	1.88 ± 0.23
		Diet	0.14 ± 0.12	1.00 ± 0.73
	100	Water	160.58 ± 44.00	29.34 ± 16.70
		Diet	6.79 ± 3.55	5.45 ± 2.51
Ag^+	**0.1**	Water	1.03 ± 0.39	2.32 ± 0.86
		Diet	0.93 ± 0.69	1.04 ± 0.11

In treatments where the leaves and medium were contaminated with the lowest AgNP concentration (1 µg L^{-1}) there was an increase in the nutrient levels, especially for NH_4, compared to control. Compared to controls, the microcosms with AgNPs had an accentuated increase of DOC in treatments where the mayflies were present, while in their absence the DOC decreased. Temperature and pH in each microcosm were constant in all treatments, with a values around 16.18 ± 0.24 ºC and pH=8.14 ± 0.02, respectively (Table S6.2).

6.3.2. Acute lethal effect of AgNPs on the invertebrate shredders and collectors

The exposure of invertebrate shredders and collectors for 96 h to AgNPs had a significant effect on their survival (repeated-measures ANOVA, $p < 0.05$). The mortality of shredders increased with increasing concentration of AgNPs and exposure time (Fig. 6.2a), while the mortality of collectors was only increased with increasing concentration of AgNPs (Fig. 6.2b). The 96 h LC_{20} (95% C.l.) of AgNPs was 45.7 (7.8 – 100.7) µg L^{-1} for the invertebrate shredder and 101.2 (96.88 - 105.7) µg L^{-1} for the collector.

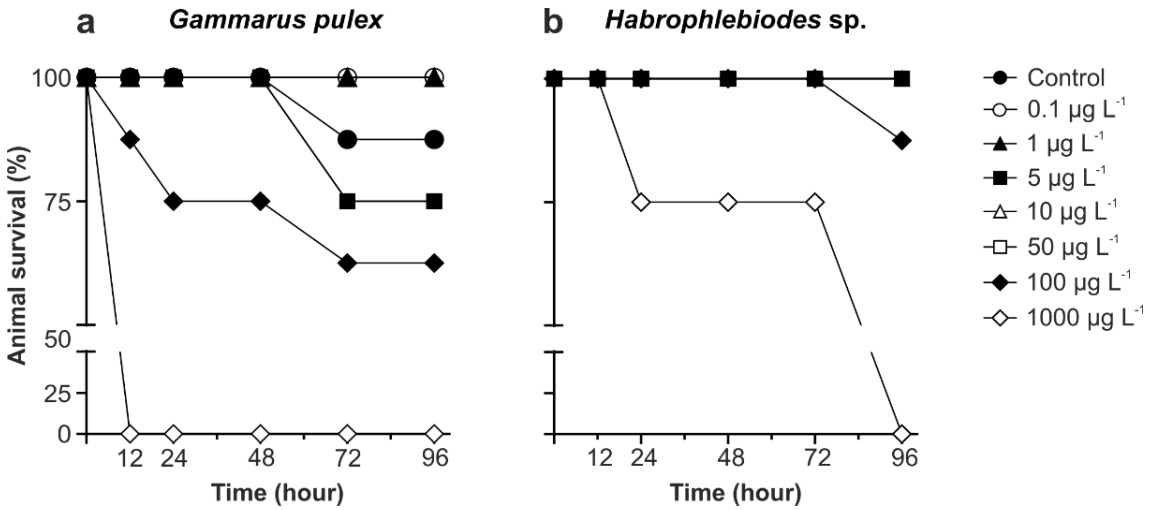


Figure 6.2 - Acute lethal toxicity of AgNPs to the invertebrate shredder *Gammarus pulex* (a) and to the collector *Habrophlebiodes* sp. (b) with respect to time.

6.3.3. Leaf decomposition and fungal activity

After 15 days, oak leaves lost 18 % of its mass in control microcosms, due to microbial decomposition. Leaf mass loss by microbes was lower in microcosms where the leaves were exposed to AgNPs and Ag^+ throught the water and also in microcosms containing leaves contaminated with Ag^+ (one-way ANOVA, $p = 0.02$; t-tests, $p < 0.05$, Fig. 6.3a). There was an effect of exposure route on leaf mass loss, which was significantly higher in leaf contaminated treatments compared to water exposed treatments (two-way ANOVA, $p = 0.007$). In control microcosms fungal biomass produced on leaves attained 668 µg ergosterol g^{-1} leaf dry mass. Fungal biomass was lower in leaves contaminated with Ag^+ and AgNPs at the highest concentration for both exposure routes (one way ANOVA, $p < 0.0001$, t-tests: $p < 0.05$, Fig. 6.3b), and the exposure route had a significant effect on fungal biomass (two-way ANOVA, $p = 0.0015$, figure 6.3b) There was a negative relationship between fungal biomass and microbial leaf mass loss after the 15 days of experiment (t-test, $p = 0.04$).

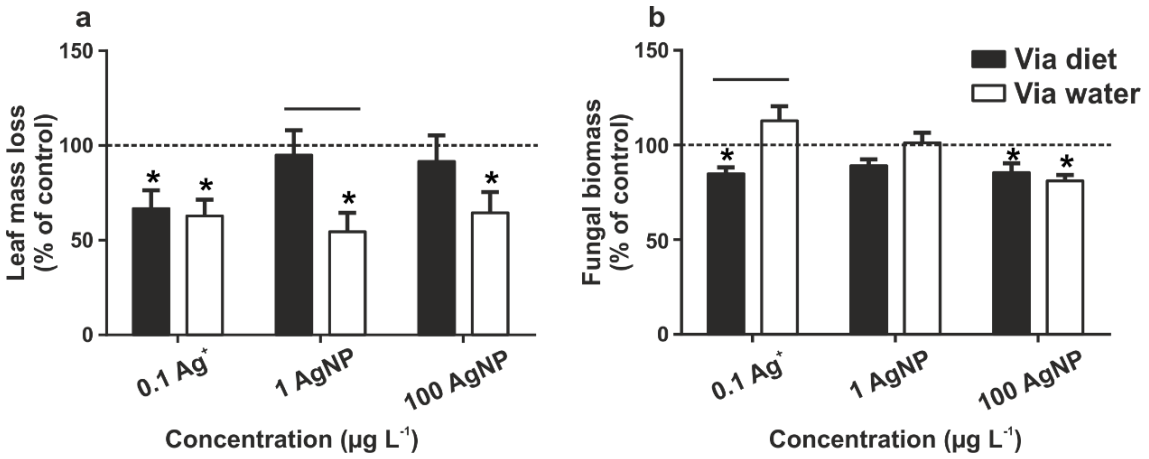


Figure 6.3 - Leaf mass loss of (a) and fungal biomass (b) on oak leaves in microcosms contaminated with AgNPs and Ag+ via water and via diet exposure (oak leaves). Results are expressed as percentage of control. Asterisks = significantly different from control (100%); dashed line = mean control line; horizontal lines = significant differences between exposure routes.

6.3.4. Leaf consumption and FPOM production by the shredder Gammarus pulex

After 15 days no significant differences in *G. pulex* wet weight was observed between control microcosms or treatments (one-way ANOVA, $p = 0.65$).

In control microcosms, consumption rates of oak leaves by G. pulex was 0.016 g leaf dry mass g^{-1} animal wet weight day^{-1}. Leaf consumption by *G. pulex* only decreased when leaves were contaminated with AgNPs at the lowest concentration (ANOVA, $p = 0.13$, t-tests: $p < 0.05$, Fig. 6.4a). However, there was an effect of exposure route on leaf consumption by *G. pulex*, which was significantly higher in water-exposed treatments compared to leaf contaminated treatments at all concentrations (two-way ANOVA, $p = 0.01$, Fig. 6.4a). Although there were no effects of AgNPs and Ag^{+} on FPOM production (Fig. 6.4b), microcosms containing mayflies had more FPOM compared to microcosms without mayflies. There was a positive correlation between leaf consumption by *G. pulex* and production of FPOM (t-test, $p < 0.01$).

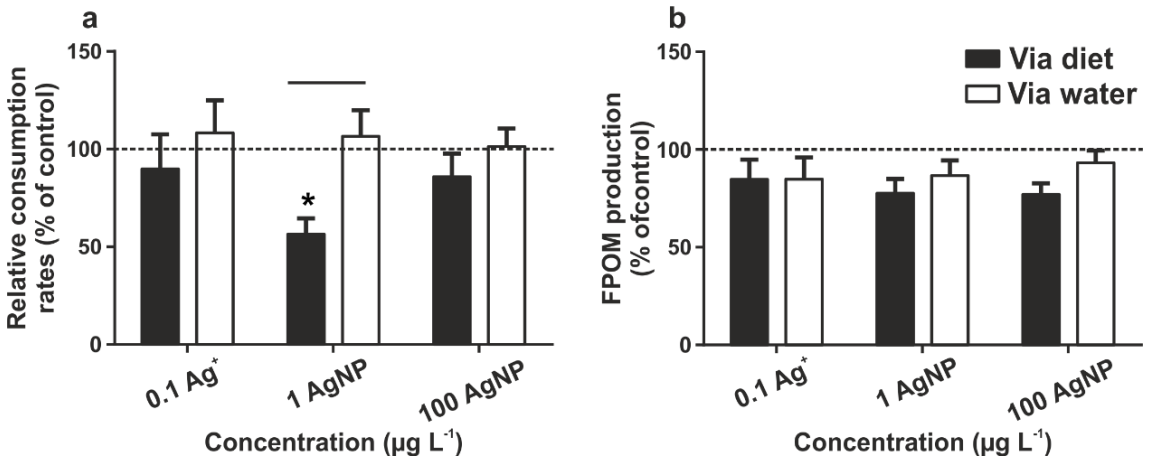


Figure 6.4 - Consumption of oak leaves (a) and FPOM production (b) by *G. pulex* exposed to AgNPs and Ag^+ via water and via diet (oak leaves) to AgNP and Ag^+. Results are expressed as percentage of control. Asterisks = significantly different from control (100%); dashed line = mean control line; horizontal lines = significant differences between exposure routes.

6.3.5. Activity of antioxidant and neuronal enzymes from invertebrates shredder and collector

The activity of GST in shredders (Fig. 6.5a) was stimulated in microcosms containing leaves contaminated with both AgNPs concentrations and in microcosms with 100 µg L^{-1} of AgNPs when supplied through the water (one-way ANOVA, $p = 0.15$; t-test, $p < 0.05$). There was a stimulation of the GST activity of collectors in microcosms containing leaves contaminated with Ag^+ (one-way ANOVA, $p = 0.014$; t-test, $p < 0.05$) and an effect of the exposure route was observed (two-way ANOVA, $p = 0.006$, figure 6.5A). CAT activity was also stimulated in shredders and collectors (Fig. 6.5b and B), especially in microcosms exposed to the lowest AgNP concentration by both exposure routes (one-way ANOVA, $p = 0.0004$ and $p = 0.08$, respectively; t-test, $p < 0.05$). However, it was only observed an effect of exposure route in CAT activity in shredders when exposed to Ag^+, which was significantly higher in water-exposed treatment compared to leaf contaminated treatments (two-way ANOVA, $p = 0.001$, figure 6.5b). There was an inhibitory effect of SOD activity only in the shredders (Fig. 6.5c) in microcosm contaminated through water with 1µg L^{-1} AgNPs, while an inhibitory effect on the ChEs activity was observed only in the mayflies (Fig. 6.5D) in microcosms containing leaves contaminated with 1 µg L^{-1} of AgNPs (one-way ANOVA, $p = 0.12$ and $p = 0.07$, respectively; t-test, $p < 0.05$).

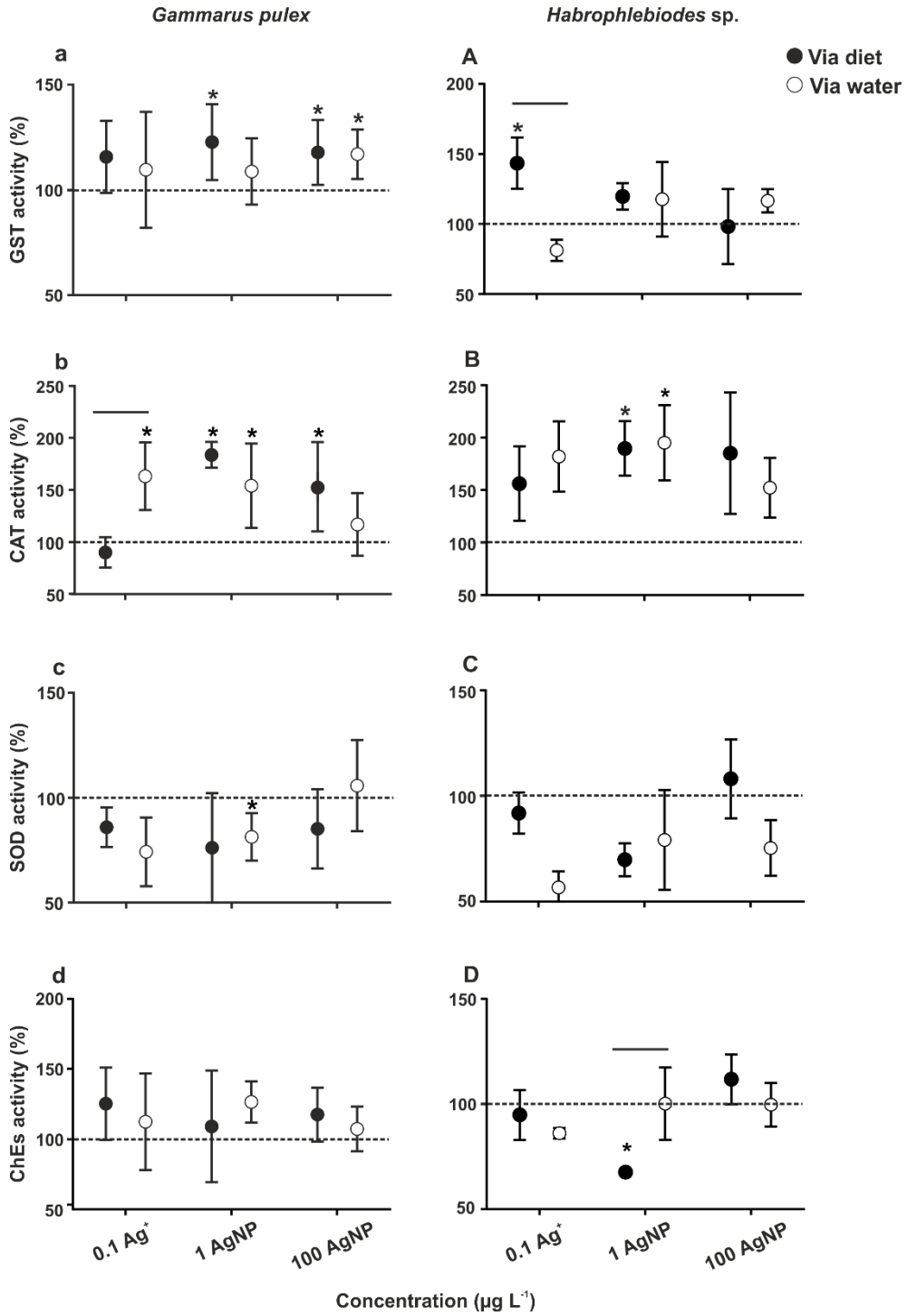


Figure 6.5 - Activities of glutathione transferase (GST; a, A), catalase (CAT; b, B), superoxide dismutase (SOD; c, C) and cholinesterases (ChEs; d, D) in shredders (a, b, c and d) and collectors (A, B, C and D) exposed to AgNPs and Ag^{+} via water and via diet (oak leaves). Results are expressed as percentage of control. Asterisks = significantly different from control (100%); dashed line = mean control line; horizontal lines = significant differences between exposure routes.

6.4.Discussion

From our knowledge this was the first successfully attempt to investigate the effects of both direct (water) and indirect (diet) exposure routes of AgNPs and $AgNO_3$ on aquatic invertebrates and consequent effects on leaf decomposition. Previous studies have evaluated the trophic transfer of AgNPs and $AgNO_3$ (algae-water fleas: Mcteer et al., 2014; shrimp-fish: Wang and Wang, 2014), but the trophic interaction studied here can be considered illustrative of detrital food web transfer, from leaf litter, to microbes, and to shredder and collector species.

Our study demonstrated that ecological effects on different functional groups of stream invertebrates vary with exposure route to AgNPs at environmentally-realistic concentrations: microbial decomposition was lower by direct exposure to AgNPs and Ag^+, leaf consumption by *G. pulex* only decreased when leaves were contaminated with AgNPs; and changes in the activity of the key antioxidant enzyme catalase indicated that both shredders and collectors were under stress caused by AgNPs and Ag^+, mainly in response to direct exposure.

6.4.1. Acute lethal effect of AgNPs on the invertebrate shredders and collectors

In our study, environmentally-realistic concentrations of AgNPs induced shredder and collector mortality during a 96h exposure period. Recently, Andrei et al. (2016) observed no mortality of *G. roeseli* when exposed to low concentrations of AgNPs (0.5-5 µg L^{-1}), while others reported a LC_{50} of 835-1000 µg L^{-1} for *G. fossarum* after 72h of AgNP exposure (Mehennaoui et al., 2016). There is no information on LC_{50} for the species of collector studied here when exposed to AgNPs. The shredders appeared to be more sensitive to AgNP exposure than the collector. Lethal effects vary among functional groups and differences in LC_{50} values depend on the inherent sensitivity of species together with factors such as life-history stage (life cycle stage: McCahon and Pascoe, 1988a; reproductive period: McCahon and Pascoe, 1988b; moult cycle stage: McCahon and Pascoe, 1988c; food availability: Heugens et al., 2006).

6.4.2. Impacts of AgNPs and Ag^+ on leaf decomposition and fungal biomass

At the end of experiment (15 days), leaf decomposition by microbes was not affected by AgNPs despite the reduction of fungal biomass in treatments were leaves were pre-exposed to AgNPs. It is possible that even when the fungal growth is inhibited, the aquatic fungi can continue performing their role, without losing their ability to decompose the leaves. On

the other hand, exposure via water severely affected leaf decomposition rates even at low AgNP concentration (1 µg L^{-1}). In these treatments, microbial communities on the leaves were constantly exposed to nano and ionic Ag stress for 15 days, which can lead to high accumulation of Ag on the leaves. This was confirmed by the high concentration of Ag found in the leaves under direct exposure. It is likely that the microbial communities did not have the opportunity for recovery, affecting the leaf decomposition process. Pradhan et al. (2011) also found that the exposure to AgNP (100 and 300 ppm) inhibited leaf decomposition and reduced microbial biomass on decomposing leaves. The higher concentrations used by Pradhan et al. (2011) cannot be compared to the ones in our study (200× higher), but it emphasize the AgNP toxicity to microbial decomposers even at low levels.

Exposure to Ag^+ inhibited leaf mass loss in both exposures routes and fungal biomass only in treatments were the leaves were pre-exposed to Ag+. In a study from Arce Funck et al. (2013a) where leaf litter was exposed to ionic Ag for 24 days, fungal biomass was reduced at 100 µg L^{-1} Ag^+ via water exposure, but there was little difference at lower concentrations (0-10 µg L^{-1}). Because fungi grow inside the leaf matrix they can be partially protected from surface metal accumulation. The concentration used in our study was very low (100× lower) compared to the one used by Arce Funck et al. (2013a), and was lower than the maximal environmental concentrations predicted in aquatic systems (0.5 µg L^{-1}; Luoma, 2008). Even so, the microbial communities were affected, supporting that Ag is one of the most toxic metals in freshwater environments (Ratte, 1999).

6.4.3. Impacts of AgNPs and Ag^+ on shredder feeding behaviour and FPOM production

We observed that the exposure route affected more the shredder feeding behaviour than the Ag form (ionic or NP) or concentration. We hypothesised that the effects of AgNPs on the shredder feeding behaviour would be more pronounced via diet exposure. In fact, leaf consumption by *G. pulex* was lower in treatments under indirect exposure to NPs compared to treatments under water exposure. Freshwater invertebrates are very sensitive to several contaminants including ionic (Arce Funck et al., 2013a) and nano (Pradhan et al., 2012, 2015) metals and the decrease in invertebrate feeding may be related to the food avoidance behaviour of shredders (Wilding and Maltby, 2006; Batista et al., 2012).

We did not expect that inhibition of consumption rates would be greatest for the lowest AgNPs concentration, especially because fungal biomass was more affected at the highest

NP concentration. Fungal conditioning of CPOM is particularly important for *G. pulex* because their consumption rates are sensitive to the biomass of fungi present in detritus. Thus, the reduction in the consumption rates was expected to be at the higher NP concentration. One possible explanation is that there was a microbial community shift in the AgNP treatments, which can lead to changes in shredder preferences. Some studies have shown that changes in fungal species composition in communities caused by chemical stress modify the palatability of leaf litter to invertebrate shredders (Bundschuh et al., 2011a; Arce Funck et al., 2016).

The reduction of the feeding rate of *G. pulex* by 50% compared to the control, at very low AgNP concentrations through dietary exposure can have consequences for the physiological fitness of the exposed gammarids (Bundschuh et al., 2011b, 2013). Moreover, this impairment is indicative of alterations in the vital ecosystem function of leaf litter decomposition (Maltby et al., 2002). *Gammarus* species play an important role in the breakdown of leaf litter (Felten et al., 2008) and are a major food resource for predators (Welton, 1979). So, effects on physiological fitness of this species might propagate to the ecosystem level: slower decomposition rates, poor quality of the leaves, lower invertebrate production and reduced energy and nutrient transfer to predators (Arce Funck et al., 2016). Our results suggest that dietary uptake of AgNPs or Ag^+ could be a route of concern in addition to direct exposure, even at environmentally realistic concentrations.

The Ag accumulation in the gammarids was more pronounced in treatments were the water was contaminated with AgNPs and Ag^+, discarding our hypothesis that a higher accumulation would be found in treatments via dietary exposure. Indeed, gammarids have an exoskeleton composed of keratin that can potentially adsorb Ag present in the water, which may explain the contradiction. A recent study by Ribeiro et al. (2016) compared the potential of *Daphnia magna* to accumulate Ag from AgNPs or $AgNO_3$ through different exposure routes (water and diet), and observed that the uptake from water explains most of the increase in Ag concentration in *D. magna* after AgNP exposure. Accumulation of Ag in animal tissues can induce toxicity not only at the organism level but also be the reason for toxicity along trophic transfer at ecosystem level, and because gammarids are a major food resource, they could be a vector of contamination in the food web (MacNeil et al., 1999).

There was a positive correlation between leaf consumption by *G. pulex* and the production of FPOM, so it was expected that less FPOM would be produced in treatments where the animals were exposed to leaves contaminated by AgNPs. On the contrary, no effects on

FPOM production by gammarids were observed. Andreï et al. (2016) observed a significant decrease in FPOM production by *G. roeseli* exposed to 0.5 µg L^{-1} of AgNPs, probably as a result of a disturbance in digestive function. FPOM is released by gammarids in form of feces and is incorporated into the aquatic food chain (Cushing et al., 1993). This important process can be indirectly altered by the presence of toxicants because when gammarids decrease their feeding activity the production of faeces and leaf detritus decrease (Dedourge-Geffard et al., 2009; Felten et al., 2008). This may have a strong impact on the detrital food chain, as faeces represent an important high quality food resource for collectors.

There have been few studies examining the effects of AgNPs and Ag^+ to collectors. However, Pristed et al. (2016) exposed a mayfly to several exposure routes of a pesticide and concluded that multiple exposure routes may increase the magnitude of effects beyond the level predicted from single route exposure. In our study, FPOM consumption proved to be an insensitive measure for determined AgNP toxicity, and a more appropriate parameter such as behaviour responses (predation or fitness: Kalcíková et al., 2014) should be used to test the effects of AgNPs in collectors.

6.4.4. Impact of AgNPs and Ag^+ on the antioxidant and neuronal enzymes of invertebrate shredders and collectors

Our study shows oxidative and neuronal stress induced by AgNPs and Ag^+ at environmental realistic concentrations in invertebrate shredders and collectors based on the activities of antioxidant enzymes, namely GST, CAT, SOD and ChEs. Other studies with metal oxide NPs have suggested that antioxidant enzymes may prevent oxidative stress at very low concentrations of CuO-NPs to other shredders (*A. ligonifer*: Pradhan et al., 2016). The levels of antioxidant enzymes of the *G. pulex* after diet (1 and 100 µg L^{-1}) and water exposure (1 µg L^{-1}) were higher than control levels for GST and CAT activities, indicating that AgNP exposure by the different routes can induce oxidative stress. Other studies found that SOD and CAT were stimulated by AgNPs via water exposure (10 µg L^{-1}, mussel: Gomes et al., 2015; 36 µg L^{-1}, snail: Ali et al., 2014). This contrasted with data from Mehennaoui et al. (2016), who found no significant alterations in the antioxidant responses when *Gammarus* where exposed to environmentally-realistic concentrations of AgNPs (1 to 3 µg L^{-1}). The authors hypothesized that the basal levels of enzyme activities may be effective to cope with the potential increased ROS levels generated by the AgNPs. An effect of exposure route was only observed in CAT activity in *G. pulex* exposed to Ag^+, which

was significantly higher in the treatment under water exposure. On the contrary, Arce Funck (2013a) found a decreased in CAT when *G. fossarum* was exposed to 0.5-1 µg L^{-1} Ag^{+} through water.

The responses of antioxidant enzymes to AgNP exposure of collectors were more visible for CAT and ChEs activities, while the stress imposed by Ag^{+} via both exposure routes was observed for GST activity. Xie and Buchwalter (2011) found that dietary exposure to cadmium, suppressed the activities of SOD and CAT in the mayfly *Centroptilum triangulifer*. SOD is the first defense against oxidative stress at a cellular level and responsible for catalyzing the dismutation of the superoxide radical O^{2-} to O_2 and H_2O_2. The induction of CAT and GST in the animals suggest the production of superoxide anions by AgNPs or an appropriate compensatory response to that production (Xie and Buchwalter, 2011), while the depletion of the antioxidant enzyme capacity like SOD suggests that the antioxidant defense system is overwhelmed by ROS and further suggests that these animals were under AgNP stress.

The mechanism of AgNPs and Ag^{+} toxicity can be differentiated by analysis of different antioxidant enzymes, as well as the collected tissues analysed in different organisms (Gomes et al. 2015; Gagné et al., 2016; Mehennaoui et al., 2016; Pradhan et al., 2016). Brittle et al. (2016) observed that Ag^{+} exposure caused the most physical damage to the neural tissue, and the hepatopancreas was identified as the best tissue indicator of AgNP pollution in crayfish. These tissues are in different locations and perform different functions, which means that each tissue may be more or less exposed to contaminants and therefore have different cellular responses. In our study, the lack of patterns in the results for ChEs activity, particularly in the shredders can be explain by the fact that we analyzed complete organisms for antioxidant enzymes concentration, not differentiating the body part from the head part (Pradhan et al., 2016).

Oxidative stress responses are generally lower at conditions of low stress (lowest concentrations) and increased at conditions of higher stress (Walters et al., 2016; Pradhan et al., 2016). The effects on enzymatic activities in the shredders and collectors were generally more pronounced at lower AgNP concentrations (1 µg L^{-1}), which was already documented in a study with terrestrial crustaceans (TiO_2 NPs, Jemec et al., 2008).

6.4.5. Unexpected effects from the presence of collectors

Unexpected indirect feedbacks were observed from the presence of collectors on *Gammarus*. Microcosms containing collectors had additional FPOM compared to

microcosms without. We hypothesized that FPOM mass would be lower in treatments with the presence of collector, assuming they would consume a considerable amount. Indeed, the increase of the metabolic activity associated with energy acquisition from food is one response of the animals when exposed to toxicants.

Collector presence did not appear to affect the consumption of leaf litter by *Gammarus*, but stimulated the GST and ChEs activities of the shredders. These can be due to non-direct animal interactions, such as misidentified predation or conspecific risk. *G. pulex* utilise leaf material as food resource, but they are known to consume prey and as well as leaf litter when both are present (Kelly et al., 2002), depending on food availability and quality. The predation risk is known to increase O_2 consumption and decrease CAT activity on damselfly larvae (Slos and Stoks, 2008). Moreover, the presence of the mayfly in the microcosms may produce some scents that can influence gammarid functions and behaviour. Sornum et al. (2012) found that the behaviour of amphipods when exposed to Cd was different in water scented by fish predators and injured conspecifics than in unpolluted water.

Only few studies have assessed the combined effects of a toxicant on shredder-collector interactions (Cd: Campos et al., 2014; molluscicide: Waller et al., 2016; TiO_2 NPs: Kalcíková et al., 2014). Shredders play a key role in leaf litter breakdown process, but mayflies are also very important not only for the autotrophic-based food web (Brittain, 1982) but also for the transfer of energy across ecosystem boundaries due to their emergence (Ballinger and Lake, 2006).

In our study, the environmentally-realistic concentrations of AgNPs and Ag^+ affected leaf decomposition driven by microbes, fungal biomass, leaf consumption by shredders and the activity of antioxidant and neuronal enzymes in shredders and collectors, with the exception for FPOM production. *Gammarus pulex* appeared to be more sensitive to AgNPs than the other organism tested. Therefore, our study highlights the importance of using detrital food webs in community ecotoxicology to assess the effects of AgNPs on species interactions and ecosystem process. The effects on leaf litter decomposition process and structure may depend on the nature of AgNP exposure. Exposure to high concentrations of AgNPs from waste-water may be brief but AgNPs have the capability to accumulate on organic matter such as the leaf litter surface. Thus, low concentrations may have stronger impacts than higher concentrations, since contaminated litter can be transported downstream or washed into unaffected riparian or floodplain habitats.

Overall our findings suggest that water quality standards should go beyond data collected from waterborne exposure, as dietary exposure is already considered as a source of NPs for higher trophic levels of aquatic organisms (Pradhan et al., 2012, 2015; Wang and Wang, 2014; Zhu et al., 2010). With this study it is clear that dietary exposure and trophic transfer of nano and ionic silver is a route of exposure that should be considered in risk assessment of AgNPs. Moreover, other factors should also be take into account, including concentrations of NPs and the physical and chemical properties of AgNPs and their potential of biomagnification along food chains.

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Chapter 7

General discussion and future perspectives

Plant litter decomposition is a process driven by a range of interacting organisms that form a detrital processing chain (Gessner et al., 2007). Microbial decomposers (aquatic fungi and bacteria) and invertebrates are involved in this process and respond differently to stress, and it is recognized that these communities are differentially sensitive to various types of pollutants (Wallace et al., 1996; Duarte et al., 2004; Batista et al., 2012; Pradhan et al., 2012, 2015a).

The few studies on the impacts of AgNPs on plant litter decomposition showed that leaf decomposition, microbial biomass and fungal reproduction are inhibited by exposure to nano and ionic Ag (Pradhan et al., 2011; Tlili et al., 2016). Unfortunately, in such studies, tested concentrations were high, so there was a lack of realism. In our study, the effects of AgNPs were assessed (1) with different NP sizes and surface charges (chapter 2); (2) at different scenarios of warming temperatures (chapter 3 and 4); (3) under environmentally realistic concentrations (chapter 5 and 6); and (4) with a special focus at the community level (fungal and bacterial communities were examined at all chapters). Moreover, the potential impacts of food as a source of metals or NPs was take into account (chapter 2 and 4), and a detritus processing chain was considered to test for possible interactions between organisms from different trophic levels from microbes, to invertebrate shredders and collectors (chapter 6).

The behavior of AgNPs in water is influenced by physical and chemical characteristics of NPs, such as particle hydrophobicity, concentration and size (Navarro et al., 2008b; Sharma et. al, 2014; Zhang et. al, 2016). In chapter 2, we assessed the impacts of AgNP size and coating on freshwater microbial decomposers of plant litter, where microbial communities associated with leaf litter were exposed for 28 days to AgNPs with 3 different sizes (100 nm PVP-dispersant, 50-60 nm and 35 nm uncoated). The exposure to nano and ionic Ag inhibited leaf decomposition and microbial activity and diversity. Fungal reproduction and diversity were the parameters more compromised even at the lowest AgNP and $AgNO_3$ concentrations. In contrast to other studies, supporting that AgNPs with smaller size are generally more toxic than those with greater size (Scown et al., 2010; Angel et al., 2013; Contreras et al., 2014; Silva et al., 2014), the 35 nm AgNP led to the lower toxicity. These NPs were uncoated and the less stable as demonstrated by the polydispersion index (PdI), leading to more aggregation. On the contrary, the larger particle size (100 nm) used in our work contained PVP as dispersant were more stable along the experiment comparing to the uncoated smaller AgNPs (35 and 50-60 nm). Particles coated with PVP and citrate are considered well-stabilized AgNPs (less aggregation) compared to the uncoated ones (Zhao

and Wang 2012b; Gomes et al., 2013). Our results strongly supported that the toxicity of AgNPs depended not only on the size and surface coating but also on the state of aggregation influenced by the environmental conditions in which AgNPs were present.

To clarify how particle size and concentration of AgNPs could affect the feeding behavior of organisms that depend on the activity of microbial decomposers, a feeding preference experiment with an invertebrate shredder was performed (Chapter 2). Freshwater invertebrate shredders preferred to feed on plant litter colonized by microbes, predominantly fungi, whose activities increase plant litter palatability for shredders (Graça, 2001; Chung and Suberkropp, 2009). Indeed, the invertebrate shredder *Limnephilus* sp. demonstrated preference for leaves exposed to AgNPs of 35 nm where we found a higher fungal diversity and biomass comparing to other treatments. On the other hand, animals avoided the leaves exposed to $AgNO_3$, where fungal diversity and biomass were severely affected. AgNP size and concentration affected the feeding behavior of shredders, indicating that the stress induced by nano and ionic Ag might have affected the invertebrate shredders directly and/or indirectly due to the effects on microbes.

The physical and chemical characteristics of the stream water (temperature, ionic strength and pH) have potential to affect NP properties (Walters et al., 2013; Siripattanakul-Ratpukdi and Fürhacker, 2014). Among physical and chemical water characteristics, temperature deserves further attention taking into account the global climate change predictions. Current climate models predict an increase in atmospheric temperatures by 2.4–6.4°C by the end of this century, as well as an increase of seasonal temperature extremes in freshwater ecosystems (International panel of climate change [IPCC], 2013). Moreover, temperature changes can have the greatest potential to mediate the effects of other more damaging stressors (Jackson et al., 2016). So, in an attempt to understand the behavior responses of AgNPs under realistic environmental scenarios, we evaluated the interactive effects of AgNPs and changes in water temperature on freshwater decomposers of leaf litter (Chapter 3 and 4). Litter-associated microbial communities were exposed to increasing concentrations of AgNPs and $AgNO_3$ at 3 temperatures: 16ºC (temperature commonly found in streams of NW Portugal in autumn); and at 10ºC and 23ºC (possible seasonal temperature extremes). As expected, the increase in temperature stimulated microbial decomposition of leaf litter and also the activity of leaf degrading enzymes, while low temperature increased fungal biomass and diversity.

Similar to that found in chapter 2, the exposure to higher AgNPs and $AgNO_3$ concentrations reduced fungal biomass, fungal sporulation and diversity. The negative effects of nano and

ionic Ag on microbial activity were more pronounced at 10 and 23ºC. On the contrary, leaf decomposition was higher in microcosms exposed to 16ºC and 23ºC, independently of the AgNP and $AgNO_3$ concentrations. Metabolic demands increase with the temperature (Brown et al., 2004), and the presence of a toxicant implies an increased in the levels of stress in the organisms. In our case, the exposure of AgNPs or $AgNO_3$ at higher temperatures was translated by higher leaf decomposition probably as a compensatory response of microbial decomposers as a mechanism to defend themselves against the stressors.

In our study, we noticed that the behavior of AgNPs was more related to water physical and chemical characteristics (pH) than to temperature. The release of dissolved Ag from AgNPs was higher only at higher concentrations, regardless the temperature. Moreover, AgNP stability (as changes in PdI and average diameter size) seemed to be more related to the increase in AgNP concentrations than to changes in temperature, despite the differences in PdI and average diameter size of AgNPs at lower concentrations at 10ºC and 23 ºC.

Chapter 3 uncovered that changes in temperature can alter the physical and chemical characteristics of the stream water. These alterations in the water characteristics can be due to dissolved organic matter (DOM) released to the water due to higher decomposition rates at higher temperatures. So, these factors influence the toxicity of AgNPs, emphasizing the importance of taking into account the interactions between environmental conditions and AgNP physiochemical properties when assessing the toxicity of nano and ionic silver.

In a follow up study (chapter 4), we also expected that the combined effect of AgNPs and changes in water temperature would have negative impacts on the ecological process in which invertebrate shredders are involved (e.g. litter decomposition, nutrient cycling), further compromising the functioning of freshwater ecosystems (Ferreira et al., 2010). So, we assessed the impacts of AgNPs and $AgNO_3$ at higher trophic levels of detrital food webs by measuring the feeding activity of the invertebrate shredder *Limnephilus* sp. and their oxidative stress responses after exposure to microbially-colonized leaves contaminated with AgNPs and $AgNO_3$ (chapter 3) at increasing temperature levels to test whether changes in temperatures modulates AgNPs effects. Moreover, the effects on these parameters were also assessed after the animals being released from the stressors (AgNPs and $AgNO_3$) and allowed to feed on non-contaminated leaves.

The increase in temperature led to a stimulation of leaf consumption by the shredders. Moreover, the higher consumption rates were not exclusively related to the increased in temperature but also to the presence of leaves contaminated with AgNPs and $AgNO_3$.

Increase in temperature is expected to increase metabolic rates of aquatic organisms (Brown et al., 2004), and the exposure to toxicants triggers energy consuming defense mechanisms (e.g. detoxification), so organisms can increase their intake of energy, to the detriment of other physiological functions (Kooijman et al., 2009). The combination of increased temperatures with chemical stressors may have led to an increase in metabolic rates, implying more energy needs, which means that the shredders in our study consumed more leaves to compensate the energy lost with the metabolism acceleration. In the pos-exposure experiment, the release from stressors (microcosms suplemented with non-contaminated leaves instead of contaminated leaves) led to higher consumption rates in animals exposed to 23ºC than to other temperatures. The higher consumption rates were not aparently caused by the release of stress and did not allow the recovery of animals activity, but major cause of stress in the animals was due to high temperatures used in our study. In a possible warming scenarios, we can expect an increase in leaf litter decomposition in the presence of shredders, which may result into a reduction of food suply in streams, affecting negatively detritus based aquatic foodwebs (Moghadam and Zimmer et al., 2016).

The most significant results were observed for the antioxidant and neuronal enzymes: AgNPs and $AgNO_3$ contamination via food induced oxidative stress in the shredder. For aquatic organisms, oxidative damage caused by AgNPs has also been reported but mainly by waterborne exposure to AgNPs (Ali et al., 2014; Walters et al., 2016). In our study, the stimulation of SOD and CAT at 10ºC for higher AgNP concentrations suggested that AgNPs leads to the production of superoxide anions. Also, the increased GST activity in the animals that fed on leaves contaminated with the higher AgNP concentrations at 16 and 23ºC, suggested an increased intracellular accumulation of ROS. It was clear that AgNP supplied via food led to negative effects on the neuronal functions of the shredders at 16ºC, as shown by an inhibition in the AChE activity. Moreover, responses of the enzymes in animals released from the stressors provided via contaminated leaves were related to the higher temperatures. This highlights the importance of considering the effects of temperature on aquatic organisms already sensitive to contaminants. The increase in temperature can induce alterations in the performance of key detritivores as those used in our study, and this may further result in altered ecosystem processing rates, including leaf litter decomposition in streams (Ferreira and Canhoto, 2015).

In the chapter 4, the enzymatic activities were correlated to the total Ag accumulated in the animal bodies, with the exception of GST that was more correlated with higher

temperatures (23ºC). The exposure to leaves contaminated with AgNPs could result in accumulation of Ag in the animals, triggering responses to toxicants at a cellular level. Being a major food source for predators, shredders are a potential vector of contamination in the food web, and the elimination of AgNPs in FPOM can induce to toxicity to organisms (e.g. collectors) in detritus food chain.

In chapter 4, we highlighted the importance of developing ecological risk assessment procedures that take into account the toxicity of AgNPs via food: the dietary exposure should be incorporated into the water quality criteria. The study helped to understand the environmental impacts associated with the exposure to AgNPs via food, providing evidences that indirect exposure to AgNPs and temperature influenced the toxicity to the shredder *Limnephilus* sp.

The effects of AgNPs have been mainly studied with single organisms (Handy et al., 2012; Dorobantu et al., 2015; Andrei et al., 2016;), which do not reflect the impacts to the entire community and associated ecological processes. Therefore, ecotoxicological studies on the environmental effects of AgNPs should consider the complexity of natural communities and ecosystems. In chapter 5, the PICT approach associated with the microbial decomposer communities was suggested as a useful ecotoxicological tool to provide more ecologically relevant information for risk assessment of AgNPs in freshwaters. Our results successfully revealed that microbial decomposer communities acquired tolerance to AgNPs and $AgNO_3$ after a chronic exposure to low concentrations of these toxicants.

For a reliable PICT detection, the choice of specific endpoints is very important to obtain accurate tolerance values (Tlili and Montuelle, 2011; Tlili et al., 2016). In our case, the microbial endpoints, namely bacterial biomass, was proven an efficient parameter to detect metal-induced tolerance of microbial decomposers. Bacterial communities exposed to AgNPs and $AgNO_3$ exhibited a higher EC_{50} than control communities reflecting community tolerance acquisition. Moreover, bacterial communities have the ability to acquire tolerance more efficiently than fungal communities, probably due the fact that bacteria have shorter life cycles than fungi, making the development of a more quick mechanisms of Ag tolerance possible. We also observed a case of co-tolerance: the bacterial community exposed to $AgNO_3$ increased their tolerance to AgNPs and vice versa. This might be explained by the similar mechanisms of detoxification of both nano and ionic forms of Ag and also by the dissolution of Ag^+.

Also some fungal species were only found in communities exposed to AgNPs and $AgNO_3$, where some species become rare. This suggests that the fungal community may have

evolved tolerance to AgNPs and $AgNO_3$: sensitive species were inhibited favoring the dominance of tolerant species (Blanck et al., 1988). In our study, we found shifts in the structure of fungal communities after 25 days of exposure to AgNPs and $AgNO_3$, similar to those found after exposure of microbial decomposers to CuONPs and AgNPs (Pradhan et al. 2011). Overall, in chapter 5 we demonstrated that toxicity studies at the community level, like PICT approaches, may provide a better understanding of mechanisms of toxicity triggered by NPs, helping to assess potential impacts of AgNPs on aquatic ecosystems.

The potential effects among trophic levels in a complex food web can be predicted by using experimental designs considering multi-trophic interactions (Englert et al., 2012; Kalcíková et al., 2014). In chapter 6, we considered a simplified detrital food web, comprising leaf litter, microbes, a shredder species (*Gammarus pulex*) and collector species (*Habrophlebiodes* sp.) and exposed them to environmentally-realistic concentrations of AgNPs or $AgNO_3$ and testing different exposure routes. From our knowledge this was the first successfully attempt to investigate the effects of both water and diet exposure routes of AgNPs and $AgNO_3$ on a food chain, comprising several trophic levels. Despite the high variability in the results, already expected given the very low tested concentrations, the environmentally-realistic concentrations caused impacts from microbes to invertebrates. Fungal biomass was mainly reduced on leaves that were exposed to higher concentrations of AgNPs, independently of the exposure route. Leaf decomposition was only affected by water exposure, probably because in these treatments microbial communities on leaves were always exposed to nano and ionic Ag stress, keeping away the opportunity of microbial communities to recover.

Consumption of leaves by *G. pulex* was lower in treatments with leaves pre-exposed to nano and ionic Ag compared to water exposure treatments, which can be related to the food avoidance behavior of shredders (Wilding and Maltby, 2006). *Gammarus* may have the capability to avoid leaves with accumulated AgNPs, as it has been shown in other studies with ionic and nano metals (Beltman et al., 1999; referenced in Batista et al., 2012). Even very low concentrations of AgNPs, supplied through diet, reduced the feeding rate of *G. pulex* by 50% compared to the control. Exposure to low concentrations to Ag^+ inhibited leaf mass loss in both exposures routes, and fungal biomass only in treatments with pre-exposed leaves, supporting that Ag is one of the most toxic metals in freshwater environments (Ratte, 1999; Wood et al., 1999).

In chapter 6, we assessed the oxidative and neuronal stress induced by AgNPs and Ag^+ in the shredders and collectors based on the activities of antioxidant enzymes, GST, CAT,

SOD and ChEs. The stimulation of GST and CAT activities was observed after exposure of shredders to AgNPs via diet or water. The response of stress enzymes to AgNPs were more visible at CAT and ChEs activity in the collectors, while the stress imposed by Ag^{+} was better noticed in GST activity. The induction of CAT and GST under AgNP exposure suggested the production of superoxide anions as a compensatory response to NPs (Xie and Buchwalter, 2011), while the reduction of the antioxidant enzyme activity, such as SOD, suggested that the antioxidant defense system could be overwhelmed by ROS.

In chapter 6, it was clear that the route by which organisms were exposed to AgNPs influenced the impacts of AgNPs on food-web components and the processes in which they are involved, highlighting the complex interactions between the organisms involved in leaf decomposition. The diet as a route of AgNPs exposure is able to induce toxicity to invertebrates and the impacts to ecological processes can be assumed even at environmentally-realistic concentrations.

AgNP characterization is considered crucial to interpret NP ecotoxicity. Ideally, AgNP properties should be characterized by more than one method and quantified in real exposure situations, taking into consideration the exposure time and the medium used in ecotoxicological assays. In our work, AgNP characterization was performed using DLS (all chapters) and NTA (chapter 6), not only in fresh medium (at the beginning of experiment) but also in water retrieved from microcosms in every medium renewal. This allowed us to check interactions between AgNPs, and AgNP and biomolecules released from the metabolism of the microorganisms. The characterization was also done for the different NP sizes (chapter 2) and in the medium exposed to different temperatures (chapter 3). The surface charge and the polydispersity index were also measured, and the accumulation of Ag in leaves, medium and animals were analyzed in all chapters.

Along the work, the toxicity of $AgNO_3$ was compared with that of Ag ions released from AgNPs to determine if the observed toxicity was due to Ag dissolution from AgNPs or was nanoparticle itself. The chronic exposure to $AgNO_3$ affected the functional endpoints in our study in distinct ways. In some cases, chronic exposure to $AgNO_3$ strongly inhibited microbial parameters, probably due to the high concentrations used (mg L^{-1}, chapter 2). On the contrary, low $AgNO_3$ concentrations (µg L^{-1}, chapter 3, 5 and 6) stimulated fungal sporulation and biomass, and leaf decomposition, which may be due to nutrient enrichment, since the ionic Ag was added as $AgNO_3$. Increased concentrations of nitrogen have been reported to stimulate both fungal and bacterial activities on decomposing leaves (Ferreira, Gulis and Graça, 2006; Fernandes et al., 2014). Another explanation relies on the fact that

at low concentrations, metals stimulate the reproduction and growth of several aquatic organisms (Batista et al., 2012; Calabrese and Blain, 2005). Fungal diversity is the only parameter that was severely inhibited by $AgNO_3$, independently of the concentration used. In our study, it seems that the toxicity towards microbial decomposers was mainly due to the nano form of AgNPs and not to the release of Ag ions from NPs: less than 1 % of dissolved Ag^+ was found in AgNP suspensions (chapter 2 and 5), and in chapter 3 the notable negative effects were observed at temperatures where dissolution of Ag in the water was reduced. AgNP toxicity to microbial decomposers may be related to AgNP absorption on the leaves, where the fungi are growing, preventing the release of spores, disabling fungal growth and even inhibiting microbial respiration. Moreover, the toxicity of $AgNO_3$ can be explained by the notable accumulation of total Ag in the water and especially on the leaves (see along the chapters), even using concentrations 10× lower than those used in the AgNP treatments.

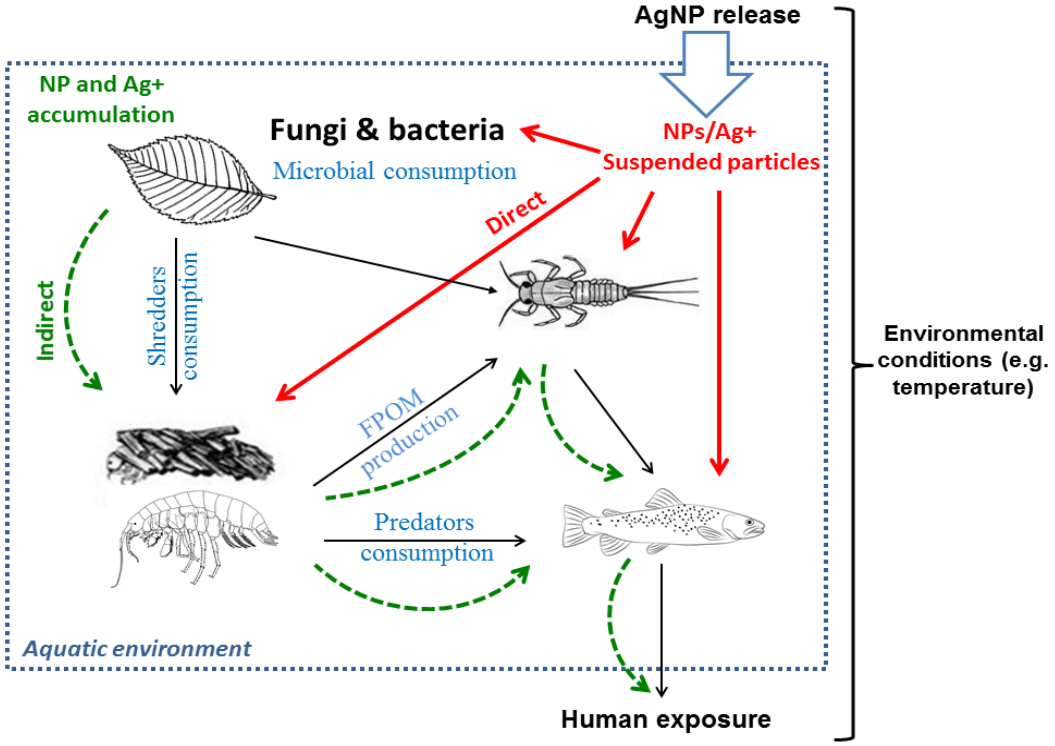

Figure 7.1 - Conceptual diagram depicting AgNPs release into the freshwater ecosystems and the potential impacts on plant litter decomposition and the organisms involved in this process. The effects on the biota can result from direct exposure (red arrows) with suspended particles in the water or from indirect exposure (green arrows) by accumulation of Ag in the leaves and in the invertebrate shredders and collectors. Environmental conditions, such as temperature, are also considered a potential factor capable to modulate the AgNP impacts on this key ecosystem process.

Figure 7.1 summarizes how AgNPs can directly and/or indirectly affect several groups of organisms involved in plant litter decomposition in freshwaters. Along this study, we observed that AgNPs could be present in the aquatic environments in their nano or ionic form released from NPs. The effects on biota can result from direct exposure of suspended particles in the water or from indirect exposure by accumulation of Ag in leaves and in invertebrate shredders.

Exposure to AgNPs from waste waters may be transitory but AgNPs have the capability to accumulate on organic matter, such as the leaf litter surface (Pradhan et al., 2015b). Shredders have a dominant role in leaf litter decomposition by transforming leaf material into material that are food resources to other organisms involved in this ecosystem process. By feeding on contaminated leaves, not only the shredders but the FPOM they produce can be transported along the stream and transferred through the detritus chain compromising organisms at higher trophic levels (Cushing et al., 1993).

Exposure to nano and ionic Ag inhibited leaf decomposition and microbial activity and diversity, with direct impact on the food choices of the invertebrate shredder *Limnephilus* sp. The effects were depended on particle size and surface coating of AgNPs, independently of the organisms tested (chapter 2). However, when environmental factors are involved the effects are not so simple to interpret. Changes in temperature influenced the characteristics of the water, which in turn change the behavior of the AgNPs and modulate the toxicity to the organisms (chapter 3 and 4).

It seems that the impacts of AgNPs were more pronounced on fungal reproduction and diversity than in other functional attributes (chapter 2 and 3). Moreover, when dealing with a higher trophic levels (invertebrate shredder and collectors), the impacts of AgNPs can be observed not only on leaf consumption but also on antioxidant and neuronal enzymes, even at environmentally-realistic concentrations (chapter 4 and 6). The exposure to leaves contaminated with AgNPs could result in accumulation of Ag in the animals, triggering responses at the cellular level. The exposure route by which the organisms tested in this study were exposed to AgNPs is also a factor contributing to the different effects observed. Results indicated that contamination by food had an impact on leaf consumption by shredders. Overall, our study encourages the use of fungal reproduction and the feeding behavior of invertebrate shredders as endpoints for assessing toxicity of AgNPs in aquatic environments. Complementing the analysis of antioxidant and neuronal enzymes in the animals is one step further to understand the mechanisms of AgNP toxicity. Additional,

studies using PICT approaches may provide a better understanding of mechanisms of toxicity triggered by NPs (chapter 5).

For a final remark, this study highlighted that the risk of AgNPs in freshwaters should be assessed taking into consideration: (1) environmentally-realistic concentrations of AgNPs; (2) physical and chemical properties and the behavior of AgNPs under different environmental conditions; (3) the dietary exposure as a source of NPs in different trophic levels in food webs; (4) the tolerance acquisition by aquatic communities; and (5) the relevance of including studies at the community level without depreciating the effects at individual level.

Taking into account the increasing production of AgNPs all over the world and in order to minimize the potential impacts of AgNP, future actions should focus on hazard and risk assessment of these nano-metals. Nowadays, risk assessment of nanomaterials is a challenge and the possible exposure of AgNPs to the environment during the entire production life cycle needs thorough attention. AgNPs may undergo agglomeration, aggregation, adhesion, diffusion, dissociation, degradation, adsorption upon release into the environment finally leading to bioaccumulation and biomagnification in trophic chains. So, for a proper risk assessment of AgNPs it is necessary to include all the factors, such as physical (size, shape, surface area and agglomeration state), chemical (chemical composition, charge and chemical reactivity), biological (route of exposure, metabolism, excretion, adduction to biological molecules) and environmental (temperature, pH, salinity, acidity, viscosity) factors. Moreover, the presence of other emerging contaminants in the environment should be taken into account for assessing the risk of AgNPs in the aquatic environments. The mixtures of contaminants in aquatic environment is a recent issue of concern, and all the factors mentioned above can also be modulated by the presence of these contaminants, influencing the toxicity of AgNPs by promoting different effects (synergistic, additive or antagonist) with impacts on freshwater ecosystems.

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Annex

Table S2.1 - Percentage contribution of each fungal sporulating species to the total conidial production on colonizing oak leaves after 28 days of exposure to increasing concentrations of coated (100 nm PVP) and uncoated (35 nm and 50 nm) AgNPs and $AgNO_3$ in microcosms (n = 3). -, not detected

Fungal taxa	Concentration (mg L^{-1})																
	Control	$AgNO_3$				35 nm				50-60 nm				100 nm PVP			
		2	5	15	25	25	50	100	200	25	50	100	200	25	50	100	200
Alatospora acuminata	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alatospora pulchella	0.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Anguillospora filiformis	0.1	-	3.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Articulospora tetracladia	53.3	38.7	18.9	-	-	66.5	63.0	54.1	19.9	31.9	41.1	35.4	25.8	20.6	36.4	27.8	27.0
***Cylindrocarpon* sp.**	-	6.7	-	-	-	-	-	7.8	1.4	-	-	-	11.6	-	-	-	-
Dimorphospora foliicola	12.1	-	3.3	-	50.0	-	-	5.5	-	-	1.0	2.9	-	-	-	-	22.2
Flagellospora curvula	3.5	-	-	50.0	50.0	-	-	-	-	-	-	-	-	-	-	-	-
F. penicillioides	-	-	-	-	-	-	-	8.2	-	-	-	-	-	-	-	-	-
***Fusarium* sp.**	-	25.4	74.4	-	-	0.6	3.3	0.5	29.4	68.1	2.0	-	-	1.7	8.3	30.6	41.3
***Infundibura* sp.**	11.1	11.4	-	-	-	14.9	31.0	18.9	40.3	-	49.0	61.7	62.6	75.6	52.3	36.1	-
Lemonniera aquatica	5.2	-	-	50.0	-	15.4	2.7	2.0	9.1	-	7.0	-	-	2.1	2.9	5.6	9.5
Lunulospora curvula	1.4	-	-	-	-	2.0	-	-	-	-	-	-	-	-	-	-	-
Tetracladium marchalianum	-	6.7	-	-	-	-	-	3.2	-	-	-	-	-	-	-	-	-
Tricospermum camelopardus	-	11.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tricladium splendens	0.1	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-
Triscelosphorus acuminatus	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-
Fontanospora eccentrica	12.2	-	-	-	-	0.3	-	-	-	-	-	-	-	-	-	-	-
Species richness	11	6	4	2	2	8	4	8	5	2	5	3	3	4	4	4	4

Table S3.1 - Percentage contribution of each fungal taxon to the total conidial production on alder leaves colonized by microbes in the stream, and then exposed or not for 21 days in microcosms to AgNPs at 10ºC, 16ºC and 23ºC. -, not detected

Fungal taxa	[AgNPs] µg L^{-1}								
	10ºC/ 16ºC/23ºC								
	0	50	250	500	1000	5000	10000	25000	75000
Alatospora acuminata	0.3/1.8/2.7	0.6/0.4/0.9	2.0/4.2/2.9	0.2/1.1/2.6	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
Alatospora pulchella	0.4/9.9/12.9	0.3/4.1/6.4	1.2/7.8/11.9	0.2/4.1/10.2	0.1/1.1/1.8	-/-/-	-/-/-	-/-/-	-/-/-
Anguillospora crassa	0.1/-/0.2	-/-/0.2	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
Articulospora tetracladia	29.1/39.5/20.3	33.2/12.5/27.2	29.3/60.8/45.5	36.2/46.6/63.9	41.6/21.9/10.3	70.6/13.4/7.6	74.6/22.0/1.3	-/-/-	35.0/5.6/-
Clavariopsis aquatica	0.9/0.5/-	0.4/0.5/0.3	1.5/0.4/0.5	0.5/0.4/2.4	0.5/-/0.1	-/-/-	-/-/-	-/-/-	-/-/-
***Cylindrocarpon* sp.**	0.8/0.8/0.2	0.4/0.4/0.4	0.1/0.4/0.3	0.2/-/0.1	0.2/-/0.2	0.1/0.2/0.4	0.3/0.5/0.7	6.6/4.8/11.1	-/13.4/-
Dimorphospora foliicola	39.5/8.6/14.9	48.2/52.3/37.3	32.8/3.1/7.9	41.4/21.4/1.3	42.0/52.1/45.9	18.8/57.0/48.4	3.9/29.0/23.4	1.9/5.4/0.7	-/57.6/47.2
Flagellospora curvula	2.0/0.3/-	0.9/-/-	3.0/-/0.2	2.2/-/0.3	0.7/-/-	0.2/0.1/-	0.3/-/-	-/-/-	-/-/-
Flagellospora penicillioides	10.6/24.3/31.6	4.5/20.7/8.4	5.9/8.5/14.1	4.1/18.8/7.2	5.0/18.1/36.6	3.5/17.0/34.4	9.5/33.8/62.6	15.4/74.5/78.3	-/23.4/25.0
Fusarium sp.	0.3/0.7/0.1	0.3/0.1/-	0.1/0.9/0.1	-/0.4/0.5	0.2/-/-	-/0.1/-	-/-/-	-/-/0.3	-/-/-
Heliscus lugdunensis	1.4/0.7/0.2	1.2/1.5/0.2	0.6/0.8/1.0	1.4/0.8/1.6	2.3/0.8/1.9	2.4/3.1/4.2	6.9/4.6/6.3	71.9/13.7/9.7	-/-/27.8
***Infundibura* sp.**	0.6/2.2/1.3	2.1/1.5/0.9	1.2/4.7/1.0	1.0/2.3/3.5	0.5/0.5/0.4	0.3/0.3/1.0	0.1/0.7/-	-/-/-	10.0/-/-
Lemmoniera aquatica	3.2/1.3/1.9	2.6/1.2/1.1	4.0/-/0.5	2.2/0.1/-	1.7/1.6/0.4	0.8/0.1/0.1	-/-/-	-/-/-	35.0/-/-
Lunulospora curvula	3.2/1.8/6.5	2.6/3.2/8.5	8.3/0.5/0.5	4.9/1.0/1.2	3.1/1.8/1.6	0.4/8.0/3.8	-/8.5/5.7	-/0.9/-	-/-/-
Tetrachaetum elegans	4.6/6.8/7.2	2.0/1.3/4.4	5.0/4.1/2.2	3.7/1.9/0.8	1.2/1.7/0.5	2.1/0.5/0.1	3.0/-/-	-/-/-	20.0/-/-

Tetracladium marchalianum	-/0.1/-	0.2/-/-	0.3/-/-	-/-/0.5	0.2/-/-	0.4/0.1/0.1	1.0/1.0/-	1.9/-/-	-/-/-
Tricladium chaetocladium	2.4/-/-	0.3/0.1/0.4	4.1/0.3/-	1.2/0.1/-	0.5/-/-	0.1/-/-	0.4/-/-	-/-/-	-/-/-
Tricladium splendens	0.1/-/-	0.2/0.1/-	0.2/-/-	0.2/-/0.1	0.2/0.1/-	0.1/-/-	-/-/-	-/-/-	-/-/-
Triscelophorus acuminatus	-/0.9/-	-/0.1/3.3	-/2.9/11.4	-/0.9/4.0	-/0.1/0.1	0.2/-/-	-/-/-	-/-/-	-/-/-
Anguillospora furtiva	-/-/-	0.1/0.1/0.1	0.5/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
Culicidospora aquatica	-/-/-	-/0.1/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
Anguillospora longissima	-/-/-	-/-/-	-/0.1/0.1	0.2/-/-	0.1/-/-	-/-/-	-/-/-	-/-/-	-/-/-
Tetracladium breve	-/-/-	-/-/-	-/0.4/-	0.3/0.2/-	-/0.1/-	-/0.1/-	-/-/-	2.4/0.2/-	-/-/-
No. of species	17/16/13	18/18/16	18/16/16	17/15/16	18/12/12	15/13/10	10/9/6	6/7/4	4/4/3

Table S3.2 - Percentage contribution of each fungal taxon to the total conidial production on alder leaves colonized by microbes in the stream, and then exposed for 21 days in microcosms to $AgNO_3$ at 10ºC, 16ºC and 23ºC. -, not detected

Fungal taxa	[$AgNO_3$] µg L^{-1}							
	10ºC/16ºC/23ºC							
	5	25	50	100	500	1000	2500	7500
Alatospora acuminata	0.4/1.5/1.7	1.8/1.5/1.4	0.1/1.9/1.0	0.7/0.6/4.0	-/0.7/3.9	-/2.2/0.9	-/0.1/-	-/-/-
Alatospora pulchella	0.4/5.4/10.0	2.3/4.8/6.1	0.1/15.1/11.7	0.3/8.0/7.9	0.1/3.2/5.4	-/5.6/4.1	-/-/-	-/-/-
Anguillospora crassa	-/-/-	0.1/-/-	0.1/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
Articulospora tetracladia	23.6/16.0/25.2	40.0/20.8/31.4	45.6/40.0/30.4	36.5/32.2/34.2	50.7/17.3/9.9	71.3/36.4/13.4	47.1/10.5/2.0	40.0/-/0.5
Clavariopsis aquatica	0.1/-/0.1	1.3/1.0/0.1	0.2/0.2/0.8	0.9/0.4/1.1	0.2/0.1/0.3	0.1/-/-	4.8/-/-	20.0/-/-
Cylindrocarpon sp.	0.3/0.2/0.4	0.1/0.5/-	0.5/0.3/-	0.1/-/-	-/0.7/-	-/0.1/0.3	-/0.5/0.5	-/3.4/2.0
Dimorphospora foliicola	60.9/47.5/23.3	34.3/51.6/12.7	43.7/15.0/21.4	36.8/36.3/19.9	33.0/51.0/56.4	15.5/34.3/51.0	13.3/48.9/62.3	-/22.2/77.9
Flagellospora curvula	0.7/-/-	3.6/0.2/-	0.4/0.2/-	1.1/-/-	0.8/-/-	0.3/0.2/-	-/-/-	-/-/-
Flagellospora penicillioides	5.4/18.5/27.9	3.8/9.9/21.7	2.0/14.7/20.4	3.3/16.1/21.8	2.9/17.3/16.4	2.8/14.5/26.2	4.8/23.1/26.5	-/47.8/9.8
Fusarium sp.	-/-/-	-/-/0.7	-/0.2/-	-/0.3/0.1	0.1/0.1/-	-/0.1/-	-/-/-	-/-/-
Heliscus lugdunensis	0.9/1.2/1.5	0.4/0.4/-	1.2/1.0/0.3	0.9/0.8/1.6	1.3/1.8/1.2	1.7/0.5/1.3	10.0/3.2/1.8	40.0/11.5/1.2
Infundibura sp.	1.3/3.1/1.5	0.6/2.2/4.3	0.6/3.5/3.5	2.4/0.8/2.8	0.6/1.2/1.8	0.3/1.2/0.8	-/0.1/0.3	-/6.9/-
Lemmoniera aquatica	1.3/1.5/0.4	3.1/1.1/0.4	1.4/0.9/0.3	2.2/0.3/0.5	2.5/1.5/1.3	1.9/1.2/0.7	-/-/0.1	-/-/-
Lunulospora curvula	2.2/2.8/2.1	3.7/2.6/6.3	1.0/2.2/1.3	3.8/1.4/1.0	2.2/4.1/2.4	1.8/2.4/0.8	16.7/13.5/6.3	-/8.1/8.6
Tetrachaetum elegans	0.9/2.1/5.1	1.7/2.3/9.0	1.5/3.6/1.7	3.7/1.4/3.1	1.9/0.3/0.6	1.6/0.7/0.3	3.3/-/0.1	-/-/-
Tetracladium marchalianum	0.1/0.1/0.3	0.1/-/-	0.1/-/0.3	-/0.1/-	-/0.1/0.3	-/0.1/0.1	-/-/-	-/-/-
Tricladium chaetocladium	1.3/-/0.1	2.8/0.6/0.3	1.3/0.5/-	7.1/0.1/-	3.6/0.4/-	2.3/0.5/0.1	-/-/-	-/-/-
Tricladium splendens	0.1/0.1/-	0.1/0.1/-	0.5/0.1/-	0.1/0.1/-	0.1/0.1/0.1	0.3/-/-	-/-/-	-/-/-
Triscelosphorus acuminatus	-/-/0.2	-/0.3/5.1	-/0.4/6.9	-/1.0/1.8	-/0.1/-	-/0.1/0.1	-/-/-	-/-/-
Culicidospora aquatica	-/-/-	-/-/-	-/-/-	-/-/-	0.1/-/-	-/-/-	-/-/-	-/-/-

Anguillospora longissima	-/-/-	-/-/-	-/-/0.1	-/0.1/-	-/-/-	-/-/-	-/-/-	-/-/-
Tetracladium breve	-/-/0.2	0.2/0.2/0.6	-/0.1/-	-/0.1/-	-/-/0.1	0.1/-/-	-/0.1/-	-/-/-
Clavatospora longibrachiata	-/-/-	0.1/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
No. of species	16/13/16	19/17/14	17/18/14	15/19/13	15/17/14	13/16/14	7/10/9	3/7/6

Table S5.1 - Physical and chemical parameters of the water collected from the microcosms (Control, NP1= 50 µg L^{-1}. NP2= 100 µg L^{-1}. NP3= 200 µg L^{-1} and AgNO3= 20 µg L^{-1}) every 5 days of renewed medium.

		Parameter								
Treatment	**Days**	**N-NO_2^- (mg L^{-1})**	**N-NO_3^- (mg L^{-1})**	**N-NH_4 (mg L^{-1})**	**TN (mg L^{-1})**	**SRP (mg L^{-1})**	**TP (mg L^{-1})**	**DOC (mg L^{-1})**	**Oxygen (mg L^{-1})**	**pH**
Control	0	0.015	0.020	0.057	1.869	0.022	0.129	54.28	6.5	5.95
NP1		0.000	0.014	0.052	1.914	0.012	0.105	52.94	6.7	6.14
NP2		0.000	0.014	0.045	1.550	0.013	0.059	55.53	6.4	6.52
NP3		0.000	0.013	0.041	1.979	0.010	0.060	54.22	6.2	6.55
$AgNO_3$		0.000	0.000	0.045	1.333	0.014	0.050	51.36	5.7	6.65
Control	5	0.005	0.006	0.020	1.160	0.013	0.064	19.23	7.9	6.49
NP1		0.005	0.006	0.021	1.377	0.015	0.080	19.31	8.1	6.62
NP2		0.005	0.006	0.022	1.293	0.017	0.091	19.80	7.8	6.66
NP3		0.006	0.009	0.049	1.256	0.018	0.101	20.46	7.7	6.69
$AgNO_3$		0.005	0.006	0.021	1.181	0.016	0.077	17.44	8.0	6.67
Control	10	0.004	0.011	0.044	0.968	0.011	0.063	14.22	7.9	6.49
NP1		0.003	0.005	0.042	1.070	0.012	0.069	15.58	8.1	6.62
NP2		0.004	0.011	0.052	1.301	0.011	0.068	15.46	7.8	6.66
NP3		0.004	0.013	0.056	1.108	0.013	0.075	16.26	7.7	6.69
$AgNO_3$		0.005	0.203	0.047	0.957	0.011	0.080	14.22	8.0	6.67
Control	15	0.004	0.007	0.046	1.435	0.012	0.060	13.55	7.4	6.60
NP1		0.004	0.012	0.042	1.007	0.012	0.061	14.02	7.4	6.56
NP2		0.004	0.004	0.028	0.980	0.011	0.066	13.32	8.4	6.62
NP3		0.004	0.035	0.068	1.070	0.013	0.065	14.38	7.5	6.67
$AgNO_3$		0.004	0.035	0.022	1.007	0.011	0.071	13.14	8.0	6.79
Control	20	0.003	0.016	0.042	0.832	0.010	0.077	13.51	7.5	6.52
NP1		0.003	0.024	0.025	0.753	0.008	0.051	14.28	6.8	6.58

NP2		0.003	0.271	0.076	0.764	0.010	0.079	13.52	7.5	6.64
NP3		0.004	0.017	0.041	0.767	0.007	0.060	13.97	6.8	6.66
$AgNO_3$		0.003	0.017	0.033	0.821	0.012	0.084	15.61	7.5	6.73
Control		0.006	0.026	0.196	2.465	0.006	0.181	11.29	7.3	7.38
NP1		0.013	0.208	0.112	2.130	0.006	0.176	14.35	7.0	7.35
NP2	25	0.010	0.149	0.106	1.801	0.009	0.131	15.23	7.5	7.35
NP3		0.012	0.305	0.051	1.412	0.005	0.039	32.85	7.4	7.36
$AgNO_3$		0.009	0.130	0.042	1.436	0.007	0.069	40.77	7.0	7.37

Table S6.1 - Physical and chemical parameters of the water collected from the streams located in the Harz Mountains, Germany.

Location		Conductivity ($\mu S\ cm^{-1}$)	O_2 dissolved ($mg\ L^{-1}$)	pH	Temp C°	SRP ($mg\ L^{-1}$)	TP ($mg\ L^{-1}$)	$N\text{-}NO_3^-$ ($mg\ L^{-1}$)	$N\text{-}NH_4$ ($mg\ L^{-1}$)	TN ($mg\ L^{-1}$)
Innerse	downstream	141	10.6	7.94	10.02	0.008	0.019	1.546	0.008	2.508
	control	176	10.4	7.94	9.94	0.004	0.018	1.265	0.011	1.970
	upstream	146	10.3	8.12	10.05	0.003	0.019	1.196	0.023	1.953
Markau	downstream	485	10.0	8.36	11.03	0.001	0.015	1.004	0.027	1.756
	control	92	10.2	8.2	10.61	0.001	0.012	0.895	0.013	1.510
	upstream	343	10.4	8.71	10.03	0.001	0.007	0.495	0.008	0.936
Oker	downstream	90	11.0	7.42	9.14	0.002	0.012	1.228	0.009	1.947
	control	73	10.1	7.35	9.17	0.006	0.014	1.775	0.012	2.661
	upstream	37	10.7	5.18	8.41	0.022	0.043	1.642	0.010	2.555

Table S6.2 - Physical and chemical parameters of the water collected from the microcosms (Control, $AgNO_3$= 0.1 µg L^{-1}, AgNP= 1 µg L^{-1} and AgNP= 100 µg L^{-1}) after 15 days of experiment. Mean ± SD, n = 10.

Treatment (µg L^{-1})		Exposure route	Parameters							
			$N\text{-}NO_3^-$ (mg L^{-1})	$N\text{-}NH_4$ (mg L^{-1})	TN (mg L^{-1})	SRP (mg L^{-1})	TP (mg L^{-1})	DOC (mg L^{-1})	Temp. (Cº)	pH
Shredder + Collector	Control		0.66±0.07	0.15±0.06	1.54±0.20	0.14±0.02	0.16±0.03	6.24±1.04	15.78±0.10	8.14±0.01
	0.1 Ag^+	Water	0.67±0.10	0.13±0.07	1.38±0.12	0.10±0.06	0.12±0.05	7.14±1.20	15.76±0.11	8.11±0.03
		Diet	0.85±0.08	0.14±0.04	1.48±0.03	0.09±0.01	0.10±0.02	6.55±1.73	16.33±0.12	8.13±0.02
	1 NP	Water	0.81±0.09	0.26±0.05	1.78±0.04	0.14±0.02	0.16±0.03	7.48±0.69	16.09±0.09	8.11±0.03
		Diet	1.00±1.01	0.29±0.01	1.89±0.05	0.17±0.04	0.17±0.02	7.77±1.67	16.19±0.13	8.08±0.03
	100 NP	Water	0.74±0.09	0.18±0.03	1.49±0.06	0.10±0.01	0.12±0.01	6.61±0.99	16.19±0.08	8.08±0.04
		Diet	0.78±0.15	0.16±0.05	1.40±0.18	0.08±0.01	0.09±0.03	6.76±1.01	16.17±0.19	8.10±0.01
Shredder	Control		0.71±0.08	0.13±0.04	1.38±0.03	0.08±0.03	0.10±0.02	7.55±1.89	16.26±0.07	8.13±0.02
	0.1 Ag^+	Water	0.81±0.07	0.09±0.02	1.45±0.17	0.09±0.02	0.10±0.02	7.05±0.49	16.10±0.28	8.21±0.01
		Diet	0.91±0.04	0.09±0.01	1.45±0.06	0.07±0.01	0.08±0.01	5.95±0.88	16.28±0.14	8.18±0.02
	1 NP	Water	0.71±0.06	0.24±0.07	1.64±0.04	0.12±0.02	0.13±0.02	6.63±1.30	16.10±0.07	8.15±0.02
		Diet	0.89±0.08	0.19±0.02	1.62±0.13	0.11±0.02	0.12±0.02	7.61±0.57	16.23±0.05	8.13±0.02
	100 NP	Water	0.74±0.06	0.16±0.04	1.52±0.09	0.11±0.02	0.12±0.02	6.71±0.39	16.46±0.12	8.08±0.02
		Diet	0.82±0.06	0.10±0.03	1.36±0.06	0.09±0.02	0.10±0.01	5.52±0.96	16.55±0.08	8.10±0.01