Universidade de Aveiro Departamento de Biologia
Ano 2013

Marco André Ferreira Fernandes

Assessment of silver nanoparticles effects: from proteins to species

Efeitos de nanopartículas de prata: das proteínas para a espécie

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Doutor António José Arsénia Nogueira Professor associado com agregação do Departamento de Biologia da Universidade de Aveiro e co-orientação científica do Doutor Rui Miguel Pinheiro Vitorino, investigador auxiliar no Departamento de Química da Universidade de Aveiro e da Doutora Maria de Fátima Tavares de Jesus, bolseira do Departamento de Biologia da Universidade de Aveiro.

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Dedico este trabalho a toda a minha família, e em especial à minha avó Flávia.

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resumo

A contínua expansão industrial de nanopartículas de prata manufacturadas, em conjugação com a sua capacidade para se libertarem de produtos de consumo, directamente para a água, irá certamente aumentar a sua acumulação no ambiente e em matrizes biológicas. O actual nível de conhecimento acerca da ecotoxicologia e destino das AgNPs em ambientes aquáticos, ainda apresenta algumas falhas. Assim, o principal objectivo desta tese, é desenvolver uma abordagem metodológica integrativa, de forma a avaliar os efeitos das AgNPs para os organismos dulçaquícolas pertencentes a diferentes níveis de organização biológica, desde parâmetros sub-individuais e sub-celulares, até individuais, extrapolando os efeitos ao nível da comunidade. Nos níveis sub-individuais e sub-celulares, estudámos as respostas bioquímicas e a expressão proteicas em *Chlamydomonas reinhardtii* expostas a AgNPs de diferentes tamanhos.

Em relação aos níveis individuais, estudámos os efeitos das AgNPs no crescimento da microalga *Pseudokirchneriella subcapitata* e os efeitos na sobrevivência, crescimento e reprodução do crustáceo *Daphnia magna* exposto a AgNPs via meio ou através da dieta. De forma a extrapolar os efeitos das AgNPs, desde os indivíduos até ao nível da comunidade utilizamos a abordagem com SSDs.

keywords Environmental Risk Assessment, biomarkers, proteomics, silver nanoparticles

abstract The continuous industrial expansion of engineered silver nanoparticles (AgNPs) in conjugation with their ability to be released from the products to the water will likely enhance their accumulation in environmental and biological matrices. The current level of understanding on the environmental fate and ecotoxicology of AgNPs in the aquatic environment still has some gaps. Thus, the main goal of this thesis is to develop an integrated methodological approach to assess the effects of AgNPs to freshwater organisms at different levels of organization, from sub-individual and sub-cellular endpoints, to individual and extrapolating to the community level effects. . At the sub-individual and sub-cellular levels we studied the biochemical and protein expression responses in *Chlamydomonas reinhardtii* exposed to AgNPs with different sizes. Concerning the individual levels we studied the effects of AgNPs in the growth of the microalgae *Pseudokirchneriella subcapitata* and the effects on the survival, growth and reproduction of the crustacean *Daphnia magna* exposed to AgNPs via the water or the diet. Finally, to extrapolate the effects of AgNPs from the individual to the community level we used the SSD approach.

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Preamble

The Industrial Revolution was a booster for the chemical industry that we currently know. The fast growth of the human population at the time increased the demand on first necessity goods, which forced chemists and engineers to respond to these growing needs and, thus, promoting the emergence of the principles of mass production to create a high variety of new chemical substances at a large scale.

Currently, the world population is seven times higher than during the Industrial Revolution period and, in overall, life expectancy increased by far. Nowadays our demand for goods never was so high, and consequently emerging technologies as nanotechnology have developed and have created several niches of market. One of them is the household product sector, where we can find a high variety of products, from anti-ageing cosmetics containing fullerenes to anti-odor clothing containing silver nanoparticles. Thus, it is imperative to evaluate the environmental impacts of these nanoproducts during their life cycle, considering their effects during production, usage and end-of-life stages.

The multidisciplinary field of Ecotoxicology and Environmental Toxicology owns integrative tools – e.g. ecological and biochemical endpoints for evaluating the effects of stressors across all levels of organization from the molecular level to whole communities and ecosystems. Tools as species sensitivity distributions for ecological risk assessment can be used to predict the no-effect concentrations of stressors and evaluate the causal relationship between stressors and effects or even to predict the likelihood of future events.

Water is an essential element to all forms of life. Freshwater is the most common type of water in inland aquatic ecosystems and, thus, is of particular interest in Ecotoxicology.

CHAPTER I: General Introduction

General introduction

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> Nanotechnology will soon revolutionize the technology field,
> bringing to light new and exotic materials and products;
> however as common there is a hiatus between
> the evaluation of the possible impacts of these nanomaterials
> on human health and environment
> and their design and commercialization. (Quik et al., 2011).

1.1 *Nanomaterials as a source of pollution*

The term "nanotechnology" was first applied in the beginning of the 70's by Professor Norio Taniguchi of Tokyo Science University to illustrate "the extension of traditional silicon machining down into regions smaller than one micron" (Edwards, 2007). However, nowadays, the widely spread and accepted definition is the engineering and production of objects by controlling shape and size at the nanometer scale (Royal Society and Royal Academy of Engineering, United Kingdom (2004) quoted by Luoma, 2008). Thus, the occurrence of the "nano" prefix in terms like nanomaterials, which include nanofilms (one dimension), nanowires, nanotubes (two dimensions) and nanoparticles (three dimensions), fills out our present vocabulary (Handy et al., 2008).

The International Organization for Standardization (ISO) defines engineered nano-objects (ENO) as objects intentionally produced, which includes particles, plates or fibers with at

least one external dimension between 1-100 nm (ISO, 2010; Mueller et al., 2012). The modern society is dependent on nanotechnology and on the related nanoproducts, which is associated with the fast growth in the development of new nanotechnologically-enabled particles, materials and products in the last few decades (Musee, 2011). Numerous products containing ENO are already on market, ranging from textiles with antibacterial properties with silver nanoparticles (AgNPs) as key component to high performance batteries with carbon nanotubes (CNT), self-cleaning paints and coatings with photocatalytically active titanium dioxide particles (nano-TiO_2) and sunscreens with zinc oxide nanoparticles (nano-ZnO) as active component (Mueller et al., 2012). Since the research on new applications of nanoparticles is increasing, it is expected that the amount of consumer products containing engineered nanomaterials (ENs) raise, representing a major shift over the presently proportion of municipal solid waste disposed globally (Walser et al., 2012). The presence of these products in disposed waste can render different properties relatively to conventional materials and potentially cause sanitary and environmental risks (Marcoux et al., 2013). So, it is mandatory an experimental prospection on the risks associated with these emerging products, in order to fulfill the demand of experimental evidences of the public and regulatory authorities (Bouillard et al., 2013). Facing the current need of information, regarding typology, distribution and exposure of ENOs, the Danish Environmental Protection Agency created the ENOs product (El Badawy et al., 2011) This will allow gathering enough data from the ENO commercialization in the Danish market and therefore permit a better quantification of the nanowaste volumes. In addition, global ENO inventories are available at the Woodrow Wilson International Centre for Scholars (http://www.nanotechproject.org/), where the company identified an increase of ENO from 212 (March 2006) to 1628 (October 2013) (Woodrow Wilson International Centre for Scholars, 2011). Moreover, accessing the Nanowerk Nanomaterial Database Inventory (http://www.nanowerk.com/) allows the association of the respective nanocomponent with the company responsible for their production and commercialization (Nanowerk Nanomaterial Database Inventory; Musee, 2011). The most common ENs in ENO are silver (383 products), followed by titanium (including titanium dioxide) as the second most referenced (179), carbon which includes fullerenes (87), followed by silicon/silica (52), zinc (including zinc oxide) (36) and gold (19) (Woodrow Wilson International Centre for Scholars, 2011).

In the present work, we focused on silver nanoparticles (AgNPs), which are particles of silver with at least one external dimension in a size range of 1–100 nm (Nowack et al., 2011). They are the most common engineered nanoparticles with a worldwide estimated production from 55 to 320 tons/year (Nowack et al., 2011; Piccinno et al., 2012). As other nanomaterials, their production and application in several areas continues to grow. AgNPs are applied in consumer products, food technology, textiles, as well as medical products and devices (Yu et al., 2013). The main applications of AgNPs are related to their antimicrobial activity, which is due to the release of ionic silver (Sotiriou and Pratsinis, 2010). The rapid growth in the commercial use of AgNPs raised concern on the increasing environmental exposure (Yu et al., 2013). In surface waters, the predicted environmental concentration (PEC) is 0.764 and 0.116 ng L^{-1}, respectively in Europe and U.S. (Gottschalk et al., 2009). The PEC is higher for the sewage treatment plant effluents: 32.9-111 ng L^{-1} and 16.4-74.7 ng L^{-1}, respectively for Europe and U.S (Gottschalk et al., 2009). In the sediment fraction is predicted an annual variation of 952 ng kg^{-1} and 195 ng kg^{-1}, respectively for Europe and U.S (Gottschalk et al., 2009).

However, aquatic organisms are not only exposed to AgNPs but also to the silver ion (Ag+), which is released from the AgNPs. Ag^{+} is recognized as being the most toxic silver form to aquatic organisms (Hogstrand and Wood, 1998) and the second following mercury among all trace metals (Rodrigues et al., 2013). Taking this in consideration allied to the high production and release of AgNPs to the environment highlights the concern on the environmental effects of AgNPs to aquatic organisms. The toxicity of AgNPs to aquatic organisms is known to be dependent on several factors such as dissolution, particle size, presence of functional groups, coatings and surface charge. (Levard et al., 2012; Ma et al., 2012).

AgNPs are known to cause deleterious effects on bacteria, fungi, microalgae, crustaceans, fish and plants (Yu et al., 2013), being classified as ‘very toxic’ to aquatic organisms (Bondarenko et al., 2013). They are most toxic to crustaceans, followed by algae, as depicted in Figure 1. Nevertheless, there is no universal agreement on the toxicity mechanisms) of AgNPs (Fabrega et al., 2011) despite several modes of action for the antibacterial activity are proposed, involving generation of reactive oxygen species (ROS), attachment and disruption of cell membrane, changes in membrane permeability, protein interaction and interference with DNA replication (Yu et al., 2013).

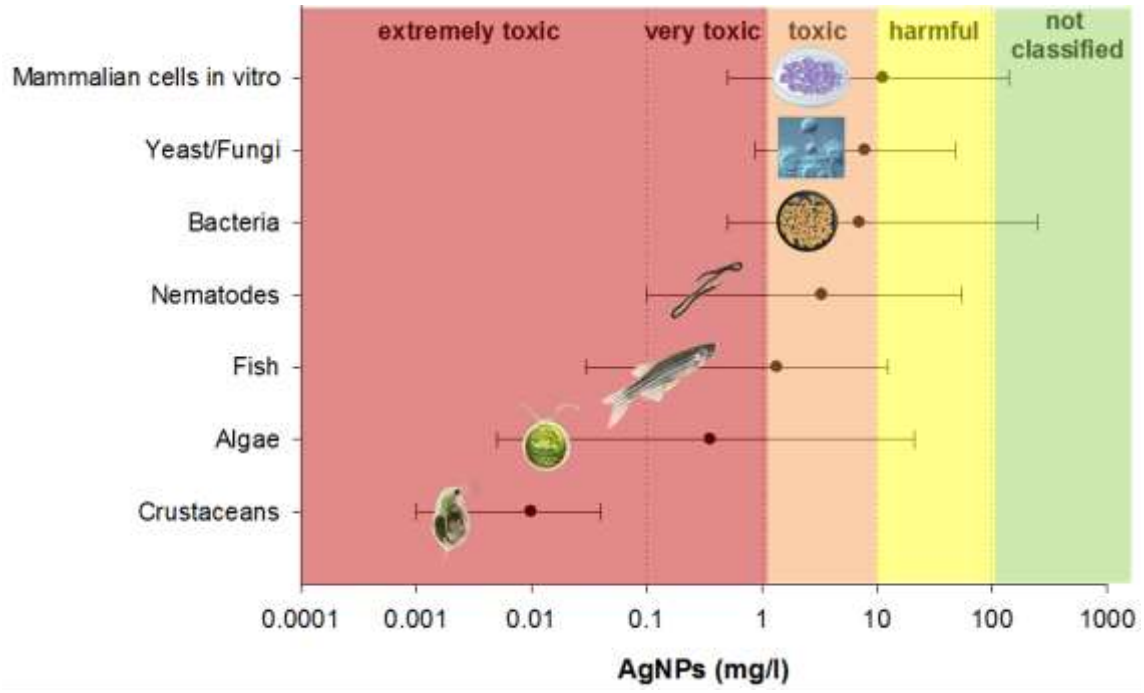

Figure 1 - Toxicity (minimum and maximum values) of AgNPs to different taxa. Presented as median L(E)C50 values for eukaryotic organisms and minimum inhibitory concentration (MIC) for bacteria. Adapted from Bondarenko et al. (2013).

1.2 *Ecotoxicological testing in freshwater ecosystems*

In the last few years, the field of Ecotoxicology was marked by the development of new assays and the emergence of novel model organisms that added relevance for the study of a particular environment. This was triggered by the research on toxic effects at different levels of biological organization (Figure 2) and the use of a set of uniform methodologies, covering a significant part of the life-cycle of an organism, enclosing both short (acute exposure) and long-term (chronic exposure) responses. Traditional endpoints provide the assessment of survival (in the case of acute exposure), sub-lethal endpoints such as growth, feeding behavior, bioaccumulation profiles and reproduction which give a measurement of fitness and therefore allow the assessment of population-level effects.

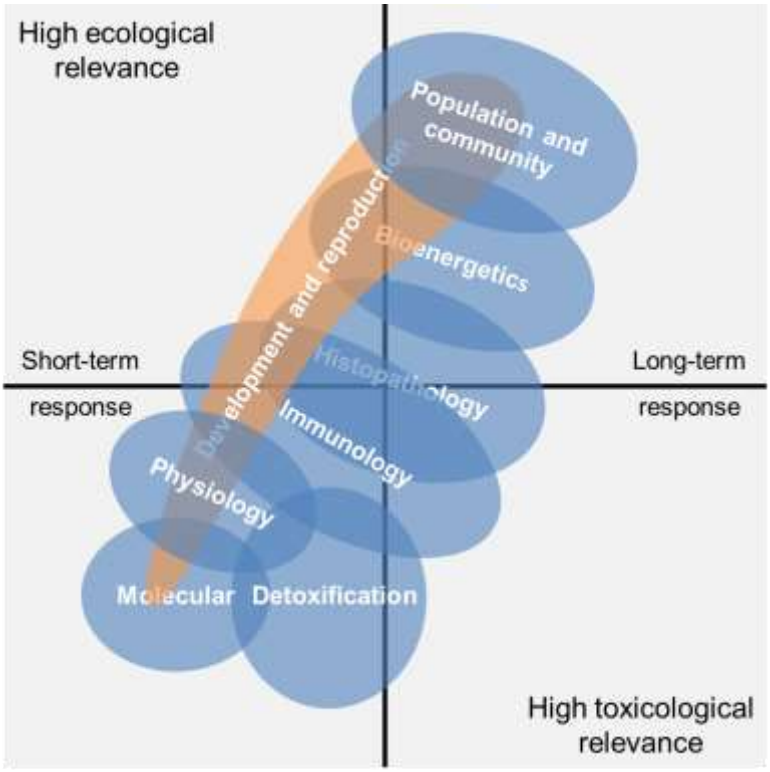

Figure 2 - Levels of biological organization, as a function of the type of response and the ecological and toxicological relevance.
Adapted from http://www.amap.no/documents/18/assessment-reports/16.

Thus, adding new and alternative endpoints at the cellular, sub-cellular, or molecular level could enhance their sensitivity, specificity and throughput capacity. Nevertheless, a good correlation is needed between sub-cellular or molecular responses and effects at higher levels of biological organization, in order to fulfill the requisites in the perspective of the Ecological Risk Assessment (ERA) policy (Posthuma et al., 2010). Furthermore, these alternative techniques can integrate responses at different levels of organization and therefore contribute for the better understanding of the mechanism behind toxic action. Consequently, they can also help in the design of predictive tools that can be employed in early warning systems (Posthuma et al. 2010).

1.3 *Endpoints at the sub-individual level*

Among the wide range of endpoints at the sub-individual level, we focused on biomarkers and proteomics.

The definition of biomarker (or biological indicator), is surrounded by a certain ambiguity changing according to the scientific area (e.g. Health sciences to Environmental sciences) and from each author perspective (Contreras et al., 2010; Nowrouzi et al., 2010). In theory, anything that can be measured in an organism can represent a marker for some biological event or process. The current definition of biomarker for Ecological Risk Assessment (ERA) relies on any biological response to an environmental stressor at a sub-individual level, measuring within organism's biochemical, molecular, genetic, immunologic, histopathological, physiological signals or even metabolites of occurred events or processes in biological systems (Huggett et al., 1992; Forbes et al., 2006; Howcroft et al., 2009). Biomarkers have the vantage of being sensitive and give an early warning of the toxic impacts to organisms, even before the ecological disturbances can be directly observed. Moreover, an improved correlation between toxic exposure concentrations and biological response is frequently observed.

Biomarkers can be studied at several levels of biological organization. Those at higher levels are supposed to integrate changes occurring at lower levels of organization (cellular or molecular). One of the most important challenges of biomarkers research is to understand the mechanisms of change at a given level and then, to understand whether and how those changes are integrated at the next higher level (Mittler, 2002; Forbes et al., 2006; Ferreira et al., 2010).

Regarding biomarkers classification, they are regularly assembled in three categories: (*i*) biomarkers of exposure – the interaction of a stressor and a target cell or molecule that is determined in certain partition of an organism, (*ii*) biomarkers of effect – a change in an organism, , that can be related with a health condition or disease and (*iii*) biomarkers of susceptibility – a specific response of an organism when exposed to a specific stressor (Huggett et al., 1992; Forbes et al., 2006).

A common consequence of most abiotic and biotic stresses is that they cause, at some stage of exposure, an increase in reactive oxygen species (ROS) (Mittler, 2002), which explains the choice for biomarkers belonging to the antioxidant defense (Figure 3).

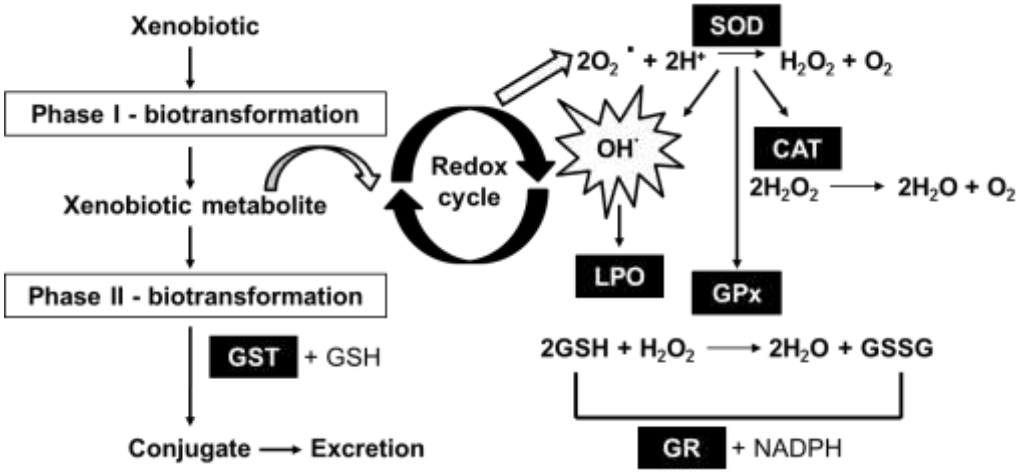

Figure 3 - Enzymatic pathways involved in xenobiotic biotransformation and antioxidant defenses (adapted from Howcroft et al., 2009). Superoxide dismutase (SOD), lipid peroxidation (LPO), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST).

Reactive oxygen species (ROS) are a wide variety of molecules and free radicals formed from molecular oxygen (O_2) (Turrens, 2003). During the normal metabolism activity, in processes as food oxidation, respiration or energy generation, O_2 undergoes tetravalent reduction to water (Turrens, 2003). However, partial reduction of O_2 results in the formation of radical species such as superoxide anion radical ($O_2^{\bullet-}$), which is the precursor of most ROS (e.g. H_2O_2, $OH^{\bullet}$) and plays a role as mediator in oxidative chain reactions (Turrens, 2003) as depicted in Figure 3. If cells are in their normal healthy state, they are able to eradicate ROS through antioxidant defenses. These antioxidant defenses include water and soluble lipids of low molecular weight, radical scavengers and specific antioxidant enzymes. (Mittler, 2002; Howcroft et al., 2009). Nevertheless, in a scenario with a high increase in ROS generation, antioxidant defenses can be overcome, resulting in oxidative damage to molecules and changes in critical cellular processes, state which is designated as oxidative stress (Mittler, 2002; Forbes et al., 2006; Howcroft et al., 2009; Ferreira et al., 2010). In this work, the following biomarkers belonging to the antioxidant defense were determined: CAT, LPO, and GST. Moreover, other biomarkers commonly used in microalgae were determined, G-Pox, and Ch *a* and *b*. Below, we provide an overall description of these biomarkers.

Catalase

Catalases (CAT) are hematin-containing enzymes that enhance the removal of H_2O_2 from the organism. The main activity of CAT is associated with the peroxisomes that function on the fatty acid metabolism (Huggett et al., 1992). There are evidences that the catalase activity is linked with the activity of glutathione peroxidase (GPx) to act against oxidative stress (Deisseroth and Dounce, 1970). The catalase function can be described by the following:

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

Guaiacol Peroxidase

Guaiacol Peroxidase **(**GPOx**)**, differently from CAT, has as reaction products, water and an activated substrate, the tetra-guaiacol (Asada, 1992), as showed by the following:

$$4\,\text{Guaiacol} + 4\,H_2O_2 \xrightarrow{GPOx} tetra - \text{guaiacol} + 8\,H_2O$$

Guaiacol is a peroxidase that some plants and algae produce. It as a function as substrate in the reduction of H_2O_2, and is oxidized by the heme co-factor of peroxidases (Amako et al., 1994). This type of peroxidases are involved in a great number of physiological processes, such as the biosynthesis and degradation of lignin, plant development and response to environmental stress such biotic stress (e.g. pathogen attack) and oxidative damage (Asada, 1992).

Glutathione S-Transferase

The glutathione S-transferase (GST) represents a family of enzymes acting as catalysts for the conjugation of various electrophilic compounds with the tri-peptide glutathione (Armstrong, 1987). They are responsible for the increase in availability of lipophilic toxicants of phase-I enzymes, acting as carrier proteins or by covalently binding to electrophilic compounds themselves reducing the probability of these compounds to bind to other (Armstrong, 1987).

Lipid Peroxidation (LPO)

Oxidative stress has a major impact on the oxidation of fatty acids (e.g. polyunsaturated) (Huggett et al., 1992). LPO can react with transition metal complexes, including the phase-I detoxification enzyme – the cytochrome P450 (Huggett et al., 1992). Several studies have verified enhancement of lipid peroxidation in several tissues due to xenobiotics or even as consequence of cellular damage (Song et al., 2010; Wang and Guan, 2010).

Photosynthetic pigments

In higher plants, light photons are captured by chlorophylls (Chl) and carotenoid pigments at the level of the thylakoid membranes in chloroplasts (Taiz and Zeiger, 2010). The photosynthetic pigments have the dual function of absorb energy to allocate for photosynthesis and to protect the photosynthetic apparatus from excess of light (Poynton et al., 2011). If excess of light occur, then a surplus of excited electrons can be produced, overcoming the capacity of the photosynthetic electron transport chain, conducting to the formation of reactive oxygen species (ROS) (Porra, 2006; Brain and Cedergreen, 2009). The use of chlorophylls as an indicator is due to their role during photosynthesis, in collecting solar energy, transforming in chemical energy (Venkatpurwar and Pokharkar, 2011) and reducing agents (NADPH or NADH). The accurate determination of Chl *a* and *b* contents and the Chl a/b ratios has been an essential tool in photosynthesis research in higher plants and green algae (Porra, 2006). The formation of ROS via exposure to trace metals is known to indirectly affect pigment synthesis (Pinto et al., 2003). Both chlorophyll and carotenoid content seems to be more sensitive as a stress indicator than the specific growth rate or even biomass (Rai et al., 2004).

1.4 *Molecular endpoints at the protein level – Proteomics*

The raise of interest on the mechanistic insight to the toxic effects of pollutants in organisms has evolved along with the advances of the molecular approaches (Martyniuk et al., 2012). The application of proteomics technologies for solving ecotoxicological issues resulted in the emergence of a new research field named “ecotoxicoproteomics” (Lemos et al., 2010). The main strengths of this area are the potential to identify early modifications

at sub-individual level in response to stressors, thus contributing to characterize their mode of action and to discover specific protein biomarkers (Lemos et al., 2010). The use of omics technologies, including quantitative proteomics methods aims to identify and quantify the dynamics of protein abundance and function, in order to gain a deeper understanding of the current biological demands.

The proteomics approach involves specific methods (Figure 4), namely the classical gel-based quantitative methods (such as two dimensional electrophoresis (2DE) and 2D differential electrophoresis (DIGE)) or the LC-based methods (such as isobaric tagging for relative and absolute quantitation (iTRAQ) (Martyniuk et al., 2012).

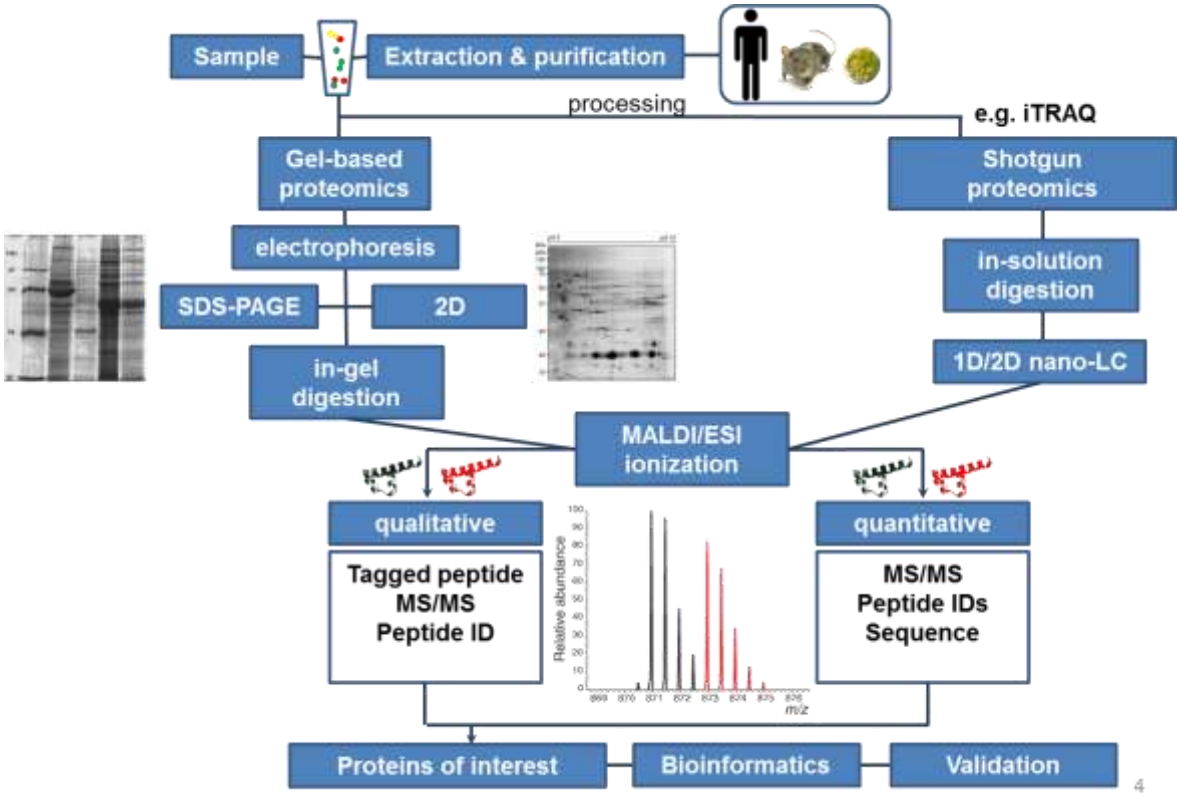

Figure 4 - General workflow of proteomic approaches

In the present work we used the iTRAQ methods (Figure 5). This method is based upon tagging the N-terminus of peptides generated from tryptic protein digests, where amine labeled peptides from different samples will have a unique isobaric tag consisting of a reporter and a mass balance (Wang et al., 2012). After cleavage, the relative intensity of reporter tags will yield information on the relative abundance of proteins in each sample,

thus retrieving the intensity of reporter ions, which will allow the relative quantification of peptides (Wang et al., 2012). This technique is advantageous because it provides reliable quantification over two orders of magnitude, allows the use of proteins of any biological system, enables the simultaneous comparison of up-to eight samples in just one experiment and increases the precursor ion intensity and reduces the sample complexity (Wang et al., 2012).

1.5 *The species sensitivity distributions approach (SSDs)*

Several approaches exist to extrapolate effects found at the individual level to effects at the whole community level, being the species sensitivity distributions (SSDs) approach the most applied. The SSDs approach was developed twenty years ago, when policy makers made pressure to researchers to optimize the use of toxicity data retrieved from chemical assays of single-species for derivation of the environmental quality criteria (EQC) (Posthuma et al., 2010), taking in consideration that risks cannot be completely eliminated but can be reduced to an acceptable low level (Newman et al., 2000). It is of common knowledge that organisms belonging to different species have different sensitivities to a certain stressor. This characteristic can be used to derive a distribution function that assumes a proportion of affected species against the concentration of the stressor, which together yields a SSD (Posthuma et al., 2010). The selection of the species set may be formed by species from a specific taxon, a species assemblage, or even a community (Posthuma et al. 2010). The SSD is derived from a set of toxicity data and visualized as a cumulative distribution function (CDF) (Figure 6).

The input data to generate the SSD, can be from acute or chronic ecotoxicity tests as E(L)C50 values. Or even values of no-observed-effect concentrations (NOECs). As convention the complementary *p* value of the 95% percentile is used to define the protective concentration of the stressor (Posthuma et al. 2010), which is designated as the hazard concentration that affect 5% of the species (HC5) (Rodrigues et al., 2013).

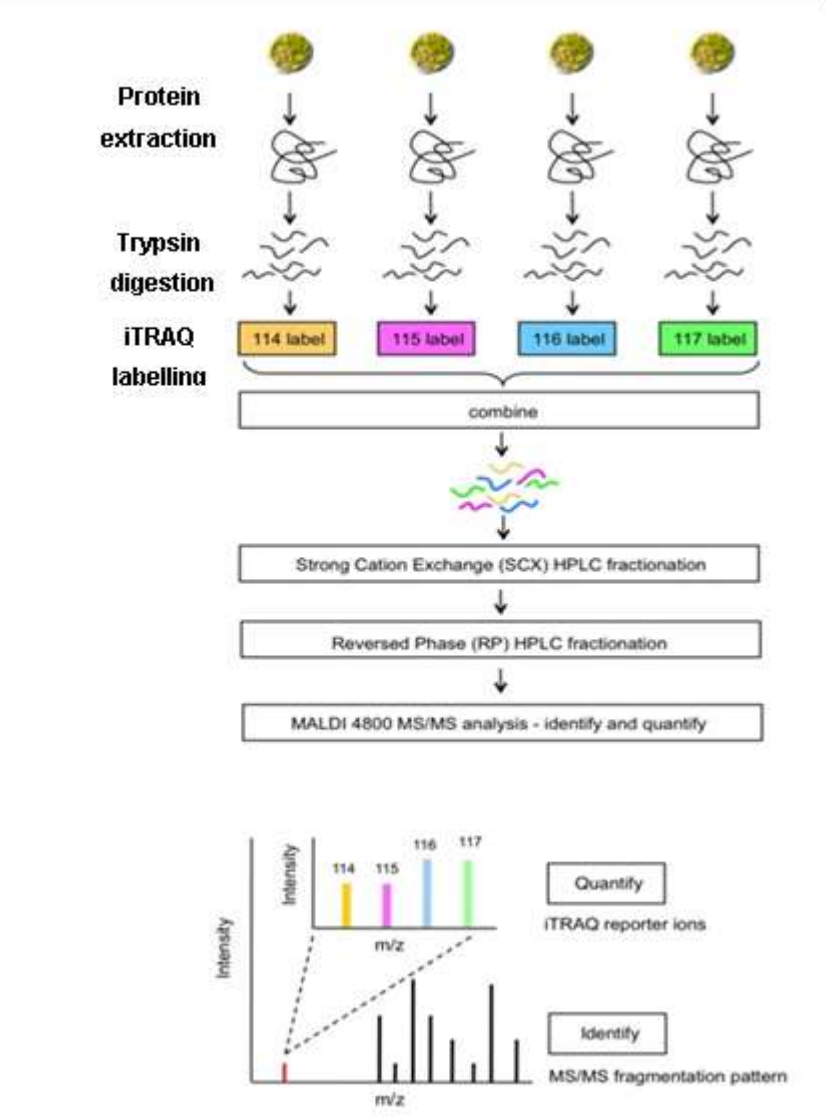

Figure 5 - iTRAQ permits the relative quantification of peptides and proteins. Both identification and quantification of peptides occurs in the MS/MS scan. Comparing the peak intensities of the iTRAQ reporter ions leads to quantification (Meyer et al., 2010).

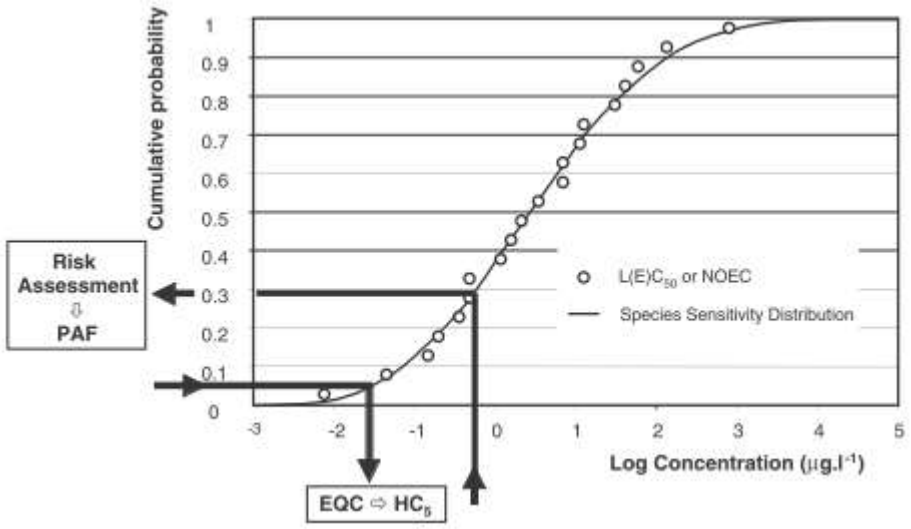

Figure 6 - A typical general SSDs, expressed as a CDF. The dots represent the input data (Posthuma et al., 2010).

1.6 *Quantitative structure–activity relationships (QSARs)*

In order to speed up the process of chemical regulation, save resources and reduce animal testing, the EU Registration system through the Evaluation, Authorization and Restriction of Chemicals (REACH) promotes the use of *in silico*-derived data to assess health and environmental risks associated with chemical exposure (Sahlin, 2013). Quantitative structure–activity relationships (QSARs) are being used as an alternative method of *in vivo* testing, where a mathematic model (parametric or non-parametric) is used to relate a set of quantitative descriptors of a chemical stressor (i.e. its physico-chemical properties) with quantitative descriptors of biological activity (Walker et al., 2003). The main purpose of QSAR methods is to identify and rationalize the involved pathways behind the mode of action of a series of chemicals and thus extrapolate for a major chemical class that shares the same physico-chemical descriptors. Ideally the QSAR models should fulfill the requirements of the OECD guidelines: (*i*) use of a defined endpoint (*ii*) described by an explicit algorithm (*iii*) having a distinct domain of applicability (*iv*) providing an appropriate measure of goodness-of-fit, robustness and predictivity (*v*) when possible give a mechanistic interpretation (OECD, 2007). Comparatively to organic and bulk chemicals, engineered nanomaterials (ENMs) renders singular properties that impose some limitations in the application of nano-QSARs. Some are related with ENMs characterization, where several methodologies and equipment's are employed, resulting in a poly-dispersive

amount of data, that needs special interpretation to make part of the physico-chemical descriptors.

The golden rule for a first approach to nano-QSARs is to limit our dataset to a highly studied nanomaterial (*e.g.* silver nanoparticles) and restrict to class of organisms (*e.g.* bacteria), and then use the gained knowledge to apply to other organism classes.

1.7 Objectives and thesis outline

The main goal of this thesis was to evaluate the effects of silver nanoparticles to freshwater organisms at different levels of organization, from sub-individual and sub-cellular endpoints, to individual and extrapolating to the community level effects. At the sub-individual and sub-cellular levels we studied the biochemical and protein expression responses in *Chlamydomonas reinhardtii*. Concerning the individual levels we studied the effects of AgNPs on the growth of the microalgae *Pseudokirchneriella subcapitata* and the effects on the growth and reproduction of the crustacean *Daphnia magna* exposed to AgNPs via water or the diet. Finally, to extrapolate the effects of AgNPs from the individual to the whole community level we used the SSD approach.

To attain the main goal, the work was divided into the following studies:

I. "General introduction", presenting and putting in context concepts and methodologies used during the experimental work;
II. "Biochemical and protein expression responses in *Chlamydomonas reinhardtii* to silver nanoparticles exposure";
III. "Chronic effects of silver nanoparticles to *Daphnia magna*: aqueous and dietary exposure";
IV. "Silver nanoparticles toxicity: species sensitivity distributions and general trends";
V. "General Discussion and Final Remarks".

Contribution to the current field of studies

This dissertation produces relevant work on the field of Ecotoxicology and Environmental Toxicology, in particular in some overlooked aspects:

- the biochemical and protein expression of microalgae in the exposure to AgNPs, which can be useful in the assessment of the mode of action and mediated toxicity of AgNPs to microalgae;
- the effects of AgNPs to the crustacean *D. magna*, concerning dietary exposure;
- the effects of AgNPs to aquatic organisms at the community level.

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CHAPTER II: Biochemical and protein expression responses in *Chlamydomonas reinhardtii* to silver nanoparticles exposure

Biochemical and protein expression responses in *Chlamydomonas reinhardtii* to silver nanoparticles exposure

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Abstract

Silver nanoparticles (AgNPs) are one of the most used nanomaterials in industrial products. However, little is known about the cellular and molecular effects of AgNPs to aquatic biota, in particular microalgae. The main goal of this study was to assess the toxicity of AgNPs to the microalgae *Chlamydomonas reinhardtii* at the phenotypic, biochemical and molecular levels. Furthermore the toxicity of two sizes (10 and 80 nm) of AgNPs was compared. *C. reinhardtii* was exposed to citrate coated AgNPs (10 and 80 nm) during 72 h. After this period the following endpoints were assessed: growth rate (GR), activity of oxidative stress enzymes, content of photosynthetic pigments and proteomic analysis of differential protein expression. The GR of *C. reinhardtii* declined after exposure to 10 and 80 nm AgNPs and followed a dose-response trend. However, the toxicity of small AgNPs was higher than that of large AgNPs: 72h-EC_{50}: 221.2±16.3 and 2508.2±282.8 µg/L, respectively. At sub-inhibitory concentrations, oxidative stress biomarkers detected modifications at low dose levels as 5µg/L, in AgNPs of 10 nm, for the chlorophylls content and glutathione S-transferase activity.

Taking in consideration the proteome response of *C. reinhardtii* to the AgNPs exposure, we found a great number of down-regulated proteins across all treatments. There are evidences that 80 nm AgNPs are more related in terms of quantitative protein response to the silver ion (Ag^{+}) treatment (added as positive control). We hypothesized that toxicity of AgNPs may be driven mostly by dissolution than by size-related effects.

Keywords: silver nanoparticles, toxicity, *C. reinhardtii*, oxidative-stress biomarkers, proteomics

Introduction

Nanotechnology soon delivered the promise of revolutionizes the technology field, bringing to light new and exotic materials and products; however a growing gap between the evaluation of the possible impacts to human health and environment and their continuous design and commercialization rise.

Silver nanoparticles (AgNPs) are particles with at least one external dimension in a size range of 1–100 nm (Nowack et al., 2011). Presently is the most common engineered nanoparticle with a worldwide estimated production of 320 tons/year (Nowack et al., 2011), where there is a continuous increase in their production and application in several areas including consumer products, food technology, textiles, as well as medical products and devices (Yu et al., 2013). This rapid growth in their commercial use raised concern on their increase in exposure into the environment (Yu et al., 2013).

AgNPs can cause deleterious effects on bacteria, fungi, microalgae, crustaceans, fish and plants (Yu et al., 2013). However, crustaceans, microalgae and fish are considered the taxa fraction more affected (Bondarenko et al., 2013). Presently, there is no universal agreement on the toxicity mechanisms of AgNPs, whereas several modes of action are proposed for the antibacterial activity, involving generation of reactive oxygen species (ROS), attachment and disruption of cell membrane, changes in membrane permeability, protein interaction and interference with DNA replication (Levard et al., 2012; Levard et al., 2013; Yu et al., 2013). Moreover, there is evidence in plants, that AgNPs can induce cell death, genotoxicity and cause DNA damage by the generation of ROS (Panda et al., 2011). Additionally, the known highly reactivity of AgNPs with hydrogen peroxide (H_2O_2) can lead to the formation of OH radicals (Lubick, 2008). Thus, biomarkers of oxidative stress are suitable to be used to evaluate early stress responses at sub-inhibitory AgNPs concentrations and consequently give insights on its mechanism of toxicity (van der Oost et al., 2003). The glutathione-S- transferase (GST) is an enzyme involved in the detoxification of xenobiotics (Oruç and Üner, 2000). Catalase (CAT) acts against oxidative stress, enhancing the H_2O_2 removal from the organism. (Barata et al., 2005). Guaiacol is a peroxidase (GPOx) that some plants and algae produce. It as a function in the reduction of H_2O_2, and is oxidized by the heme co-factor of peroxidases (Amako et al., 1994). Oxidative stress has a major impact on the oxidation of fatty acids (Huggett et al., 1992).

Lipid Peroxidases (LPO) can react with transition metal complexes, as cytochrome P450 (Huggett et al., 1992). Several studies have reported promoted of lipid peroxidation due to xenobiotics or even as consequence of cellular damage (Song et al., 2010; Wang and Guan, 2010). The ROS formation through exposure to trace metals is known to indirectly affect pigment synthesis (Pinto et al., 2003), therefore chlorophyll content can be used as a stress indicator (Rai et al., 2004).

High-throughput technologies such as mass spectrometry can provide an in-deep evaluation of the health status of an organism or even a community, and auxiliary in the elucidation of a particular mode of action of a chemical stressor (Lemos et al., 2010).

The aim of this study was to evaluate using both phenotypic, biochemical and molecular endpoints the toxicity of two sizes of citrate coated AgNPs (10 and 80 nm) to the microalgae *Chlamydomonas reinhardtii*.

Material and methods

2.1 Reagents

All reagents were supplied by Sigma-Aldrich (St. Louis, MO, USA) with the highest degree of purity available, unless otherwise stated. The iTRAQ Reagent-8Plex kit was supplied by Applied Biosystems (Foster City, CA, USA). Citrate coated silver nanoparticles with sizes of 10 and 80 nm (nominal size provided by manufacturer) were acquired from NanoComposix with a stock concentration of 20 mg L^{-1}.

2.2 Nanoparticles characterization

STEM pictures

STEM (HR-FESEM Hitachi SU-70, Hitachi High-Tech) operating at 30.0 kV was used to visualize particle size, shape and aggregation state in samples of nanoparticles suspensions. Prepared suspensions of AgNPs in Woods Hole MBL medium (Stein and Hellebust, 1980) at 1.0 mg/L were left to settle for 24 h and then placed on a copper grid support for posterior visualization. Individual measurements of particles diameter were made in ImageJ (v1.47, NIH) for 153 (10 nm) and 294 (80 nm) particles.

Dynamic light scattering (DLS)

Particle size distribution was determined by dynamic light scattering (DLS) using a Zetasizer Nanoseries (Malvern Instruments, USA) with a 633-nm laser source and a detection angle of 173º (capable of detect particles from 1 nm to 10 mm). Measurements were conducted using a 1 ml sample deposited in a disposable polystyrene cuvette. Zeta potential was measured using a disposable electrophoretic flow-through cell with an internal volume of approximately 0.9 ml. These measurements were performed in MBL medium at 1 mg/l of AgNPs. In order to determine AgNPs changes over time we performed these measurements after 1, 24, 48 and 72 h of exposure.

Microalgae culture conditions

C. reinhardtii was cultured in unialgal batch cultures with sterilized Woods Hole MBL medium (Stein and Hellebust, 1980) at 20±1 ºC, under continuous and uniform cool-white light (4000 lux) and continuous aeration.

2.3. Microalgae assays

Two independent assays were performed with *C. reinhardtii*: (a) exposure to both 10 and 80 nm AgNPs for the determination of the growth inhibition; (b) exposure to sub-inhibitory AgNPs concentrations (derived from *a*), in order to assess stress biomarkers and protein expression profiles.

(a) The algae growth inhibition tests were based on the OECD guideline 201 with an adaptation for 24 wells microplates (OECD, 2006). Tests started with 1.0×10E4 cells/ml (in log exponential growth phase) in MBL medium and carried out at 24±1 ºC for 72 with the same light cycle and luminous flux of the culture conditions. Range finding assays were indicative of a great difference of sensitivities between 10 and 80 nm AgNPs. Therefore different concentrations intervals were chosen for AgNPs exposures: 75 – 500 µg/L (10 nm) and 75 – 2500 µg/L (80 nm). Cell density was determined by optical density (OD) at 440 nm (Jenway 6505 UV/Visible spectrophotometer, UK) and converted to cell number employing the linear regression model previously developed for this specie (Cells/ml = 7.0E6×OD – 3.0E4; r^2=0.986).

(b) The same conditions were applied as in (*a*) but in this case assays ran in 10% pre-saturated (relatively to the exposure concentrations) 50 mL glass vials with AgNPs and were incubated in an orbital shaker at 150 rpm. *C. reinhardtii* with 5.0×10E4 cells/ml (in log exponential growth phase) was exposed in triplicate to 2 – 80 µg/L (10 nm) and 31 – 1200 µg/L (80 nm) AgNPs. At the end of the exposure period of 72 h, cell density was determined (as described above) and samples were harvested by centrifugation for 10 min at 3000×g and 4 ºC. Then the pellet was immediately frozen in liquid nitrogen and stored at −80 °C until biomarkers and protein analyses.

2.4. Biomarkers

On the day of enzymatic analyses, samples were defrosted on ice, homogenized at 4 °C in 1 ml of K-phosphate buffer 0.05 M (pH 7.8) containing 0.2% (v/v) Triton X-100 according to Olmos et al. (1994) using a sonicator (KIKA Labortechnik U2005 ControlTM) and centrifuged for 20 min at 10,000×g to separate the post-mitochondrial supernatant (PMS) (Howcroft et al., 2011). Enzymatic determinations were made spectrophotometrically (Thermo Scientific Multiskan Spectrum) using 96 wells microplates. Enzymatic activity was determined in quadruplicate and expressed as nanomoles of substrate hydrolysed per minute per mg of protein. Protein concentration was determined by the Bradford method (Bradford, 1976), at 595 nm, using γ-globulin as a standard.

Catalase (CAT)

CAT activity, using 15 µl of PMS was measured at 240 nm by monitoring (for 3 min) the decrease in absorbance due to degradation of H_2O_2, as described by Claiborne (1985).

Guaiacol peroxidase (GPOx)

GPOx activity, using 25 µL of PMS was measured using a method described by Cakmak and Marschner (1992) with minor modifications. Briefly, a reaction mixture consisting of 17 mM H_2O_2 and 2% guaiacol was assayed for 10 min and the activity was measured as the appearance of tetra-guaiacol at $\lambda = 470$ nm.

Lipid peroxidase (LPO)

The level of lipid peroxidation products was assayed following the method described by Heath and Packer (1968) with some modifications by measuring thiobarbituric acid-reactive substances (TBARS). The reaction included a mixture of 300 µl homogenized sample, 1 mL trichloroacetic acid sodium salt (TCA) 12% (w/v), 1 mL 2-thiobarbituric acid 0.73% (w/v) and 800 µl Tris–HCl 60 mM with diethylenetriaminepentaacetic acid (DTPA) 0.1 mM. The reaction mixture was then incubated at 100 °C in a water bath for 1 h. After, quickly cooled down in an ice

bath, samples were centrifuged for 10 min at 10,000×g. The absorbance of the supernatant was read at 532 nm and was applied a correction for unspecific turbidity by subtracting the absorbance measured at 600 nm. LPO was expressed as nmol TBARS hydrolysed per minute per mg of protein.

Glutathione S-Transferase (GST)

GST activity, using 100 µl of PMS was determined at 340 nm by monitoring the increase in absorbance (for 5 min) following the general methodology described by (Habig et al., 1974) with modifications introduced by Frasco and Guilhermino (2002). Activity determinations were made using sample and 200 µl of the reaction mixture (10 mM reduced glutathione and 60 mM 1-chloro- 2.4-dinitrobenzene in K-phosphate buffer (100 mM, pH 6.5).

Pigment extraction and quantification

Samples were sonicated with an ultrasonic probe at level 5 to 6 (50–60 W) for 20s (Branson Sonifier-450) in 80 % acetone in an ice bath. Then samples were centrifuged for 10 min at 3000×g and 4 ºC. After centrifugation, the supernatant was immediately used for pigment quantification (Schagerl and Kunzl, 2007).

Chl *a* and *b* absorbance's (Abs) were determined spectrophotometry (Jenway 6505 UV/Visible spectrophotometer, UK) at 663, 646 and 647 nm, and Chl *a* and *b* concentrations were derived by the following equations: [Chl *a* (µg/mL)] = 12.25 × Abs(663) – 2.55 × Abs(647); [Chl *b* (µg/mL)] = 20.31 × Abs(646) – 4.91 × Abs(663) (Porra et al., 1989).

2.5. Protein expression

A. Protein extraction and quantitation

Samples were defrosted on ice, homogenized in a probe sonicator in 1 ml phosphate buffer (pH 7.4) with 1% (v/v) Triton X-100 at 4 °C. After centrifugation at 10,000×g for 10 min, the resultant supernatants were quantified in terms of protein content by the Bradford method (Bradford, 1976), at 595 nm using BSA as standard.

B. Gel-free approach - iTRAQ

1. Protein digestion and labelling with iTRAQ reagents

A pre-step of in-solution protein digestion was applied for the iTRAQ labelling methodology where 100 µg of protein of each sample was mixed with triethyl ammonium bicarbonate buffer (TEAB) (1 M, pH 8.5) and 2% SDS to achieve a final concentration of 0.5 M and 0.05 %, respectively (Vitorino et al., 2012; Alves et al., 2013).

A reducing step was performed, adding 50 mM tris (2-carboxyethyl) phosphine (TCEP) to samples and incubating at 60 ºC during 1 h. Then, samples were alkylated with 10 mM S-methyl methanethiosulfonate (MMTS) at ambient temperature during 10 min (Vitorino et al., 2012; Alves et al., 2013). Hereafter, three micrograms of trypsin were added to each sample and was allowed to incubate at 37 ºC during 18 h. Samples were allowed to dry in a SpeedVac (Thermo Savant) (Vitorino et al., 2012; Alves et al., 2013).

Digested sample peptides were labelled with the iTRAQ® reagent - 8plex (AB Sciex, Framingham, MA). Briefly, peptides were reconstituted in 70 % ethanol/ 30 % TEAB 0.5M, added to each label and carried out for 2 h at room temperature. Water was added to stop the reaction. After all, the five 8-plex experiments were mixed, acidified with formic acid and dried using SpeedVac (Vitorino et al., 2012; Alves et al., 2013).

2. Protein identification and quantification by 2D-LC-MS/MS

Labelled peptides were separated by a multidimensional LC approach based on a first dimension with a C18 reverse phase HPLC column (as previously described by Manadas et al., 2009; Alves et al., 2013) and a second dimension with the acidic reverse-phase system.

Sample loading was performed at 200 µL/min with buffers (A) 2% ammonium hydroxide and 0.014% formic acid in water, pH 10 and (B) 2% ammonium hydroxide and 10% acetonitrile (Van Donk et al.) in water, pH 10. After 5 min of sample loading and washing, peptide fractionation was performed with linear gradient to 70 % B over 85 min. Sixty fractions were collected, dried, and ressuspended in 2 % ACN, 0.05 % trifluoroacetic acid (TFA). Collected fractions were then separated as previously described by Alves et al. (2013). In brief, peptides were loaded onto a C18 pre-column (5 µm particle size, 5 mm, Dionex) connected to a reverse-phase column PepMap100 C18 (150 mm × 75 µm I.D., 3 µm particle size). The flow-rate was set at 300 nL/min. The mobile phases A and B were 2% ACN, 0.05% TFA in water and 90 % ACN, 0.045 % TFA, respectively. The separation was monitored at 214 nm using a UV detector (Dionex/LC Packings, Sunnyvale, CA). Using the micro-collector Probot (Dionex/LC Packings) and, after a lag-time of 5 min, peptides eluting from the capillary column were mixed with a continuous flow of α-CHCA matrix solution were directly deposited onto the LC-MALDI plates.

3. LC-based ID

The spectra were generated and processed with 4800 MALDI-TOF/TOF^{TM} (Applied Biosystems) and analysed by the Mascot software (v.2.3.0.2, Matrix Science Ltd). Protein identification based on MS/MS data were performed with SwissProt protein database (release date 01012011, all Green Plant categories). Default search parameters were used: specifying TrypChymo as the digestion enzyme and 2 missed cleavages, fixed modification of iTRAQ 8Plex and 40 ppm tolerance A filter of $p<0.1$ was applied and quantitative normalization was performed in relation to the sample control.

2.6. Statistical analysis

Statistical analysis were performed in SigmaPlot (version 11.0, Systat Software Inc.), unless otherwise stated.

A one-way ANOVA was used to detect the differences between groups for normally distributed data sets. When data did not pass the Kolmogorov–Smirnov normality test and the Levene's homogeneity of variance test, a Kruskal–Wallis test was used. However, if

significant results were found, either the Dunnett or Dunn's test was used to detect significant differences between treatment and control groups. The effect concentration (EC's) toxicity values were calculated using a non-linear allosteric decay function in an in-built Microsoft Excel spreadsheet. To test statistical dependence between variables, Spearman rank order correlation was used. All statistical analyses were based on at least 0.05 significance level.

Tree clustering of single linkage was done with Euclidean distances in STATISTICA (version 8.0, StatSoft, Inc.). Heat map matrix was built using Matrix2png with $\log_2$ data (Pavlidis and Noble, 2003).

2.7. Gene network pathway analysis

UniProt ID's of identified proteins were mapped into gene identifiers with the ID mapping tool (http://www.uniprot.org/jobs/). Gene Ontology (GO) annotations were analyzed with AmiGO the Gene Ontology Classification System (database version 1.8, http://amigo.geneontology.org/) to identify biological functional annotations, followed by slim down associations with Plant GO slim**.** Ontology selection as enrichment analysis was done by right- side hyper-geometric statistic test and its probability value was corrected by the bonferroni method. We used the STRING software (Szklarczyk et al., 2011) that builds functional protein-association networks based on compiled available experimental evidence.

Results and Discussion

Nanoparticles characterization

The concentration of AgNPs was 1 mg/L in the stock solution. The particle concentration for the 10 and 80 nm AgNPs was 2.98×10^{13} and 4.34×10^{11} particles/L, respectively. STEM pictures of AgNPs (Figure 7) in test media showed a disagreement between the mean sizes values provided by manufacture and our study, a variation of two-fold (18.28±6.11 nm, N=153) for the 10 nm particles was found. The 80 nm particles were in the size range provided by manufacture, relatively to ours measurements (74.87±13.35 nm, N=294). This may be due to manufacture measurements were made in ultrapure water, a non-relevant medium for ecotoxicology studies. Measurements of AgNPs sizes in test media using STEM (Figure 7) and DLS (Figure 8) showed no significant correlation at 24 h. Nevertheless, DLS is still a good tool for measuring particles stability in media by characterizing the surface charge of particles, through zeta potential measurements over the time of experiments, which along with hydrodynamic diameter can give the notion of agglomeration and aggregation state of particles. Moreover, we verified that in absolute terms, for both particles the mean size of AgNPs increased during exposure whereas stability decreased (Figure 8).

STEM

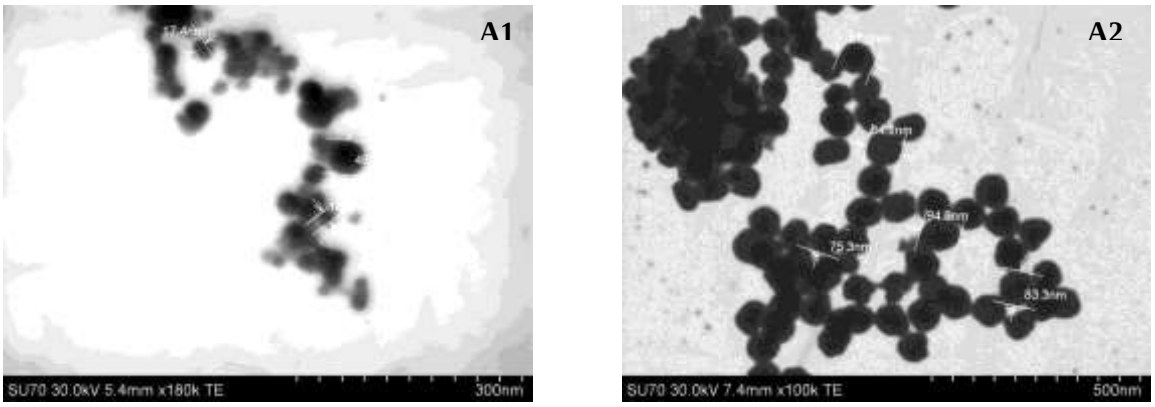

Figure 7 - STEM pictures of citrate coated AgNPs in MBL medium after 24 h: 10 (A1) and 80 (A2) nm.

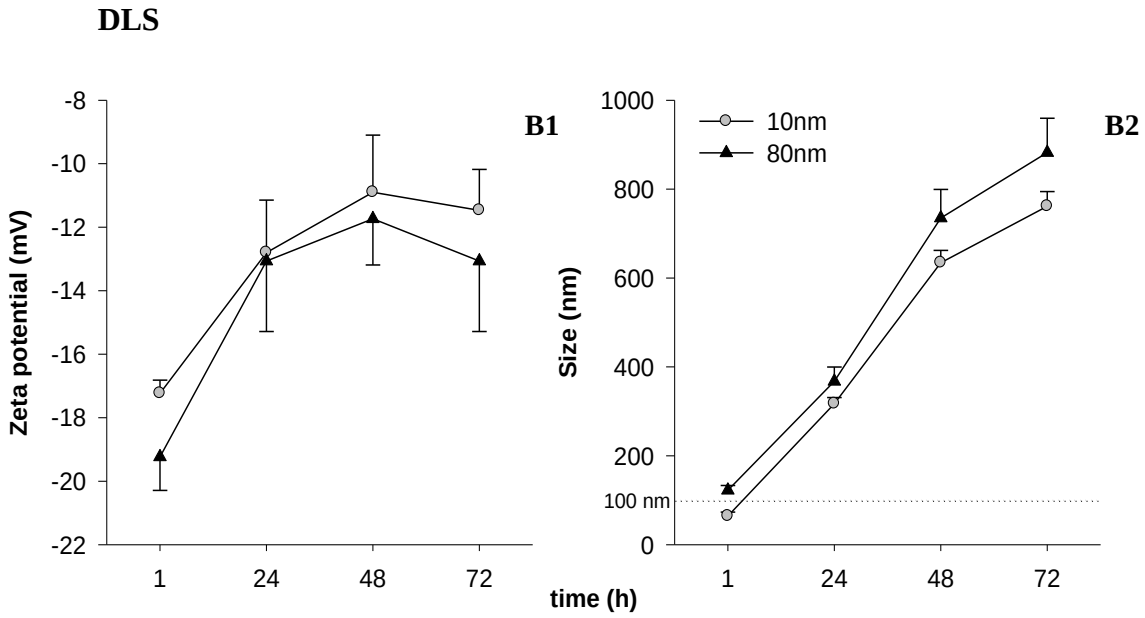

Figure 8 - DLS measurements of zeta potential (B1) and size (B2) of citrate coated AgNPs in MBL media: 10 (grey filled dots) and 80 (black filled triangles) nm for exposure times from 1 to 72 h. Dotted line as reference for the 100 nm size.

C. reinhardtii growth inhibition

Concerning the growth endpoint, *C. reinhardtii* was more sensitive for the 10 nm AgNPs (Figure 9), almost 11-fold than AgNPs of 80 nm. The EC_{50} value of 80 nm AgNPs had to be extrapolated by a dose-response curve: 2508.2±282.8 µg/L. The 10 nm particle followed a dose response trend, with an EC_{50} value of 221.2±16.3 µg/L. The LOEC values for both nanoparticles were in the range of 75 µg AgNPs/L.

The 96h-EC_{50} value derided from grow inhibition of *Pseudokirchneriella. subcapitata* exposed to AgNPs coated with metal-oxide of primary size of 26.6 nm was 190 µg/L (Griffitt et al., 2008). Oukarroum et al. (2012) observed a 50% decrease in total chlorophyll content when exposed *Chlorella vulgaris* for 24 h to 10 mg/L of uncoated AgNPs with 50 nm. The lowest observed inhibition concentration 96h-IC_{50}, retrieved from literature was found in *P. subcapitata* for the chlorophyll *a* endpoint exposed to 5µg/L of uncoated AgNPs of 25.4 nm (McLaughlin and Bonzongo, 2012). However, taking in consideration only the growth endpoint for freshwater microalgae, the lowest EC_{50} reported in literature was at the level of 33.79 µg/L in paraffin coated AgNPs with a size range of 3-8 nm (Ribeiro et al., 2014). Assessing the toxicity of a stressor based only in

one endpoint is too reducing, so we also evaluated a set of sub-cellular and molecular endpoints. In order to achieve that, we have used sub-inhibitory AgNPs concentrations for *C. reinhardtii*, derived from the growth endpoint (highest concentration was the EC_{20} values) of both AgNPs sizes for testing biomarkers of exposure (Figure 10, 11 and 12).

Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and the anti-oxidant defenses which eliminate ROS before damage can occur (Smith et al., 2013).

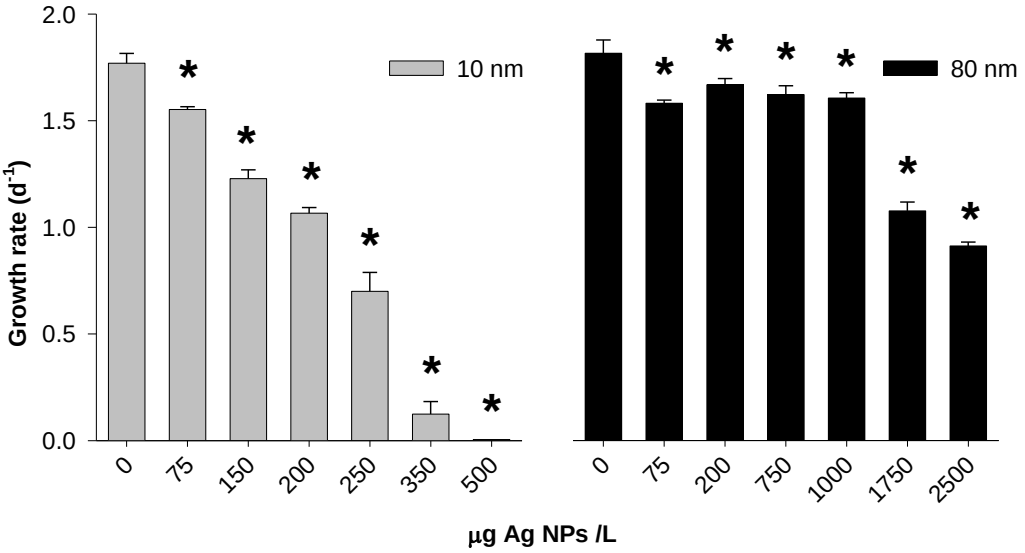

Figure 9 - Growth rate of *C. reinhardtii* exposed to AgNPs: grey bars (10 nm) and black bars (80 nm). Comparisons were made with control group (0). Data are reported as mean ± SE Dunn's method) *$p < 0.05$.

Biomarkers

The biomarkers have been used as a tool to detect early signs of chemical effects that can pose risk to aquatic ecosystems (Domingues et al., 2010). All biomarkers tested were significantly affected by the 10 nm particles (at least by one concentration), whereas only GST was affected by 80 nm AgNPs (Figure 10 and 11). For the 10 nm AgNPs, both GST and Chl *a*+*b* were sensitive for concentrations as low as 5 µg/L (Figure 10). Follow-on biomarkers dependence (Table 1) for the 10 nm particles, significant negative correlation was found, between Chl *a*+*b* with GST and G-Pox. For all others was found a positive significant correlation, reaching its maximum between LPO and GST biomarkers (Table

1). In opposite, both CAT and GST were negatively significant correlated in the 80 nm particles (Table 1).

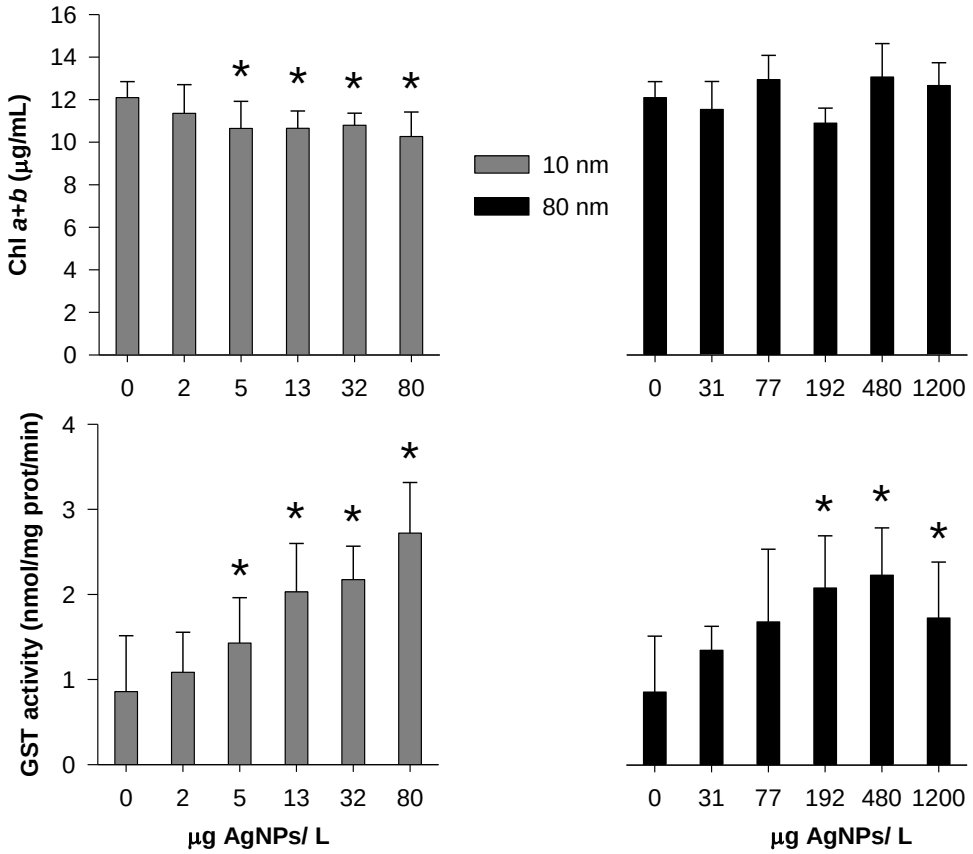

Figure 10 - Biochemical response of *C. reinhardtii* to AgNPs: grey bars (10 nm) and black bars (80 nm). Chlorophyll *a* and *b* (Chl *a*+*b*) and glutathione S-transferase (GST). Bars are mean values and the corresponding standard error bars. *= Dunnett's test, $p<0.05$.

The lack of biochemical markers data for AgNPs exposure for microalgae and even plant species is a major constrain for a reliable assessment of the associated risks that this nanomaterial can pose. Nevertheless, taking in consideration animal exposure, we can verify that CAT and GST activities were increased in carp exposed to citrate coated AgNPs in liver and gills tissue (Lee et al., 2012). LPO was induced in *Elliptio complanata* digestive glands at 4 and 0.8 µg/L, respectively for 20 and 80 nm citrate coated AgNPs (Gagné et al., 2013). In overall (Gagné et al., 2013) stated that the effects measured

through biochemical markers (metallothioneins, protein-ubiquitin and DNA damage) of the 80 nm particles were more closely related with Ag^{+} than with 20 nm AgNPs.

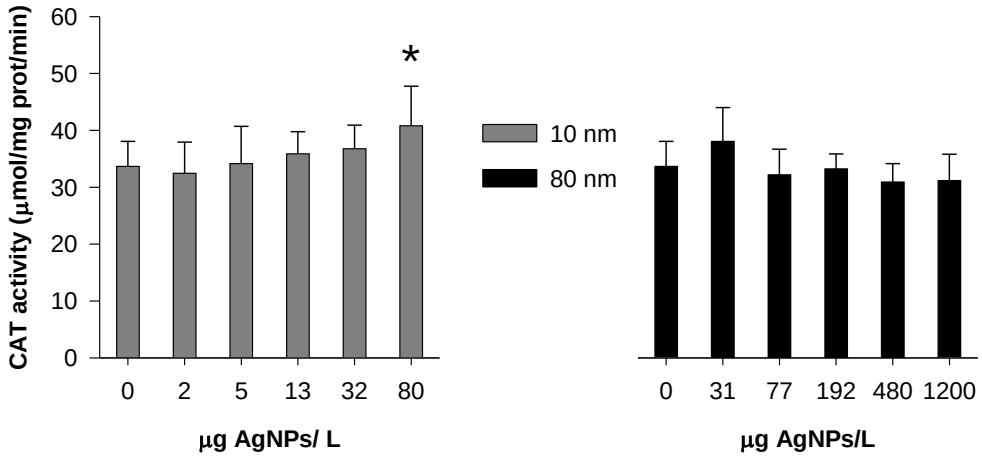

Figure 11 – Catalase activity (CAT) of *C. reinhardtii* to AgNPs: grey bars (10 nm) and black bars (80 nm). Bars are mean values and the corresponding standard error bars. *= Dunnett's test, $p<0.05$.

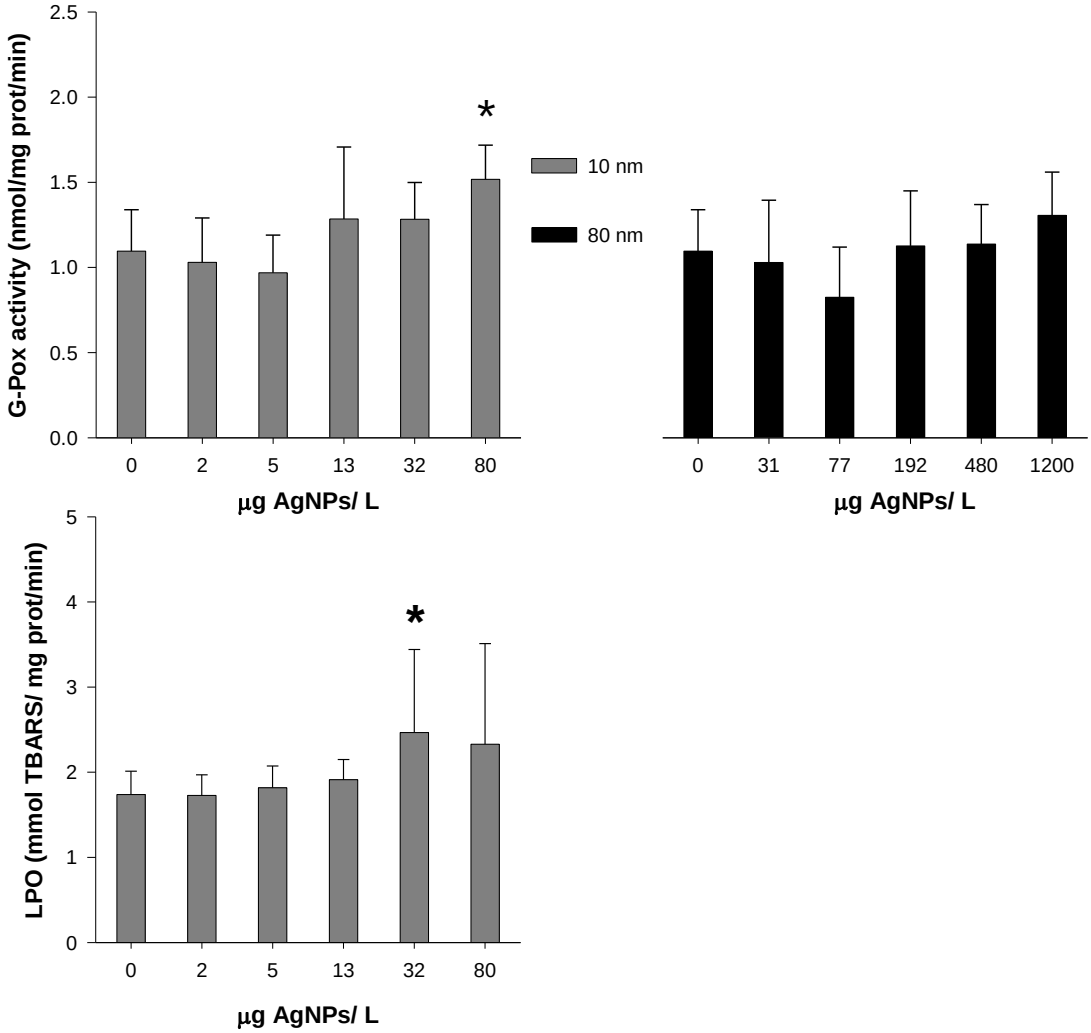

Figure 12 - Biochemical response of *C. reinhardtii* to AgNPs: grey bars (10 nm) and black bars (80 nm). Presented as guaiacol peroxidase (G-Pox) and lipid peroxidase (LPO). Bars are mean values and the corresponding standard error bars. *= Dunnett's test, $p<0.05$.

Table 1 - Spearman correlation between the biomarkers tested for AgNPs 10 nm exposure. *p<0.05; ** p<0.001.

	10 nm		80 nm	
	GST	**G-Pox**	**GST**	
Chl *a*+*b*	-0.28 *	-0.25 *	**CAT**	-0.26 *
CAT	0.41 **	0.32 *		
LPO	0.47 **	0.30 *		
G-POx	0.42 **			

The response of *C. reinhardtii* proteome to the treatments with AgNPs and Ag^+ are shown in Figure 13 and the associated biological functions in Figure 14. From the identified proteins (68), quantitative changes were found in 26, where we can observe two distinct major groups ([A] and [B to E]) based on the cut-off at 2.8 in the Euclidean distance (Figure 13A1). The AgNPs concentrations of 31 and 480 µg/L within the 80 nm particles present the major resemblance. Taking in consideration the tree clustering, the Ag^+ treatment seems to be more associated with the 80 nm-AgNPs.

In terms of protein expression, the treatment with 80 nm particles at 480 µg/L has shown more up-regulated proteins, followed by Ag^+ and 10 nm particles at 31 µg/L (Figure 13A2). Briefly, at 480 µg/L of the 80 nm-AgNPs, was found the following up-regulated proteins: P25840, heat-shock 70 kDa protein (HSP70); Q3ZJ24, elongation factor Tu (EF-Tu) and Q9FR95, arabidiol synthase (AtPEN1) (presented as accession number, name and acronym). The HSP70 have been applied as a non-specific stress detector caused by trace metals as cadmium (Tukaj et al., 2011). The EF-TU proteins are multifunctional, playing an important role in protein biosynthesis and in acting as chaperones by preventing other proteins to aggregate during abiotic stresses (Fu et al., 2012). The AtPEN1 is an enzyme with function in biosynthesis, being responsible for converting oxidosqualene to arabidiol, and for adding some minor products as the 20, 21-epoxide (Xiang et al., 2006). For the Ag^+ and 10 nm-AgNPs at 31 µg/L, we found as up-regulated, the same last two proteins as in the 80 nm-AgNPs at 480 µg/L. In both AgNPs at minor concentrations, we found only the AtPEN1 up-regulated. In contrast, most of the proteins were found down-regulated across all treatments (Figure 13A2).

Taking in consideration differences at the dose level, within AgNPs treatments we can found for both higher concentrations of 10 and 80 nm a slight increase in the number of up-regulated proteins relatively to the corresponding particle at low doses (Figure 13A2). In order to clarify the role of the differently expressed proteins, a qualitative analysis was performed in terms of functional clusters. As can be observed in Figure 14, according to the AmiGO classification system, identified proteins are distributed in 36 different biological function categories being the top six, the cellular processes (14.75%), metabolic process (13.52%), the nucleobase-containing compound metabolic process (5.74%), cellular component organization (5.33%), transport (4.92%) and response to stress (4.51%). Filtering the annotation of response to stress (GO: 0006950) by the annotation of response to silver ion (GO: 0010272), employing the slimmer tool of the AmiGO classification system, we obtained the following related proteins (↓ down-regulated and ↑ up-regulated): ↓F4I7I0, Alanine aminotransferase 1, mitochondrial; ↓ Q9LXC9, Soluble inorganic pyrophosphatase 1, chloroplastic; ↓ P29197, Chaperonin CPN60, mitochondrial; ↓ Q84MA2, Type I inositol 1,4,5-trisphosphate 5-phosphatase 1; ↓ Q9SJ66, Probable sucrose-phosphatase 2; ↓ P46602, Homeobox-leucine zipper protein HAT3; ↓ Q9LKZ3, Retinoblastoma-related protein 1; ↑ Q9FR95, Arabidiol synthase. The Q9SJ66, Q9LKZ3 and Q9LJF5 proteins had the lowest down-regulation values, with a median of -3.8, -6.0 and -5.3-fold change related to treatment control.

In summary, both AgNPs sizes were able to induce inhibition effects to the grow rate of *C. reinhardtii*, in which the 10 nm AgNPs was the more toxic. The 10 nm AgNPs treatments have shown an increase in oxidative stress On the other hand the 80 nm AgNPs at the highest concentration had more up-regulated proteins.

The dissolution *per se* can't explain the increased oxidative stress obtained through the measurement of biochemical markers in the 10 nm particles, suggesting a provable interaction with the surface of microalgae cells, as also pondered by (Gagné et al., 2013).

Protein expression profiles

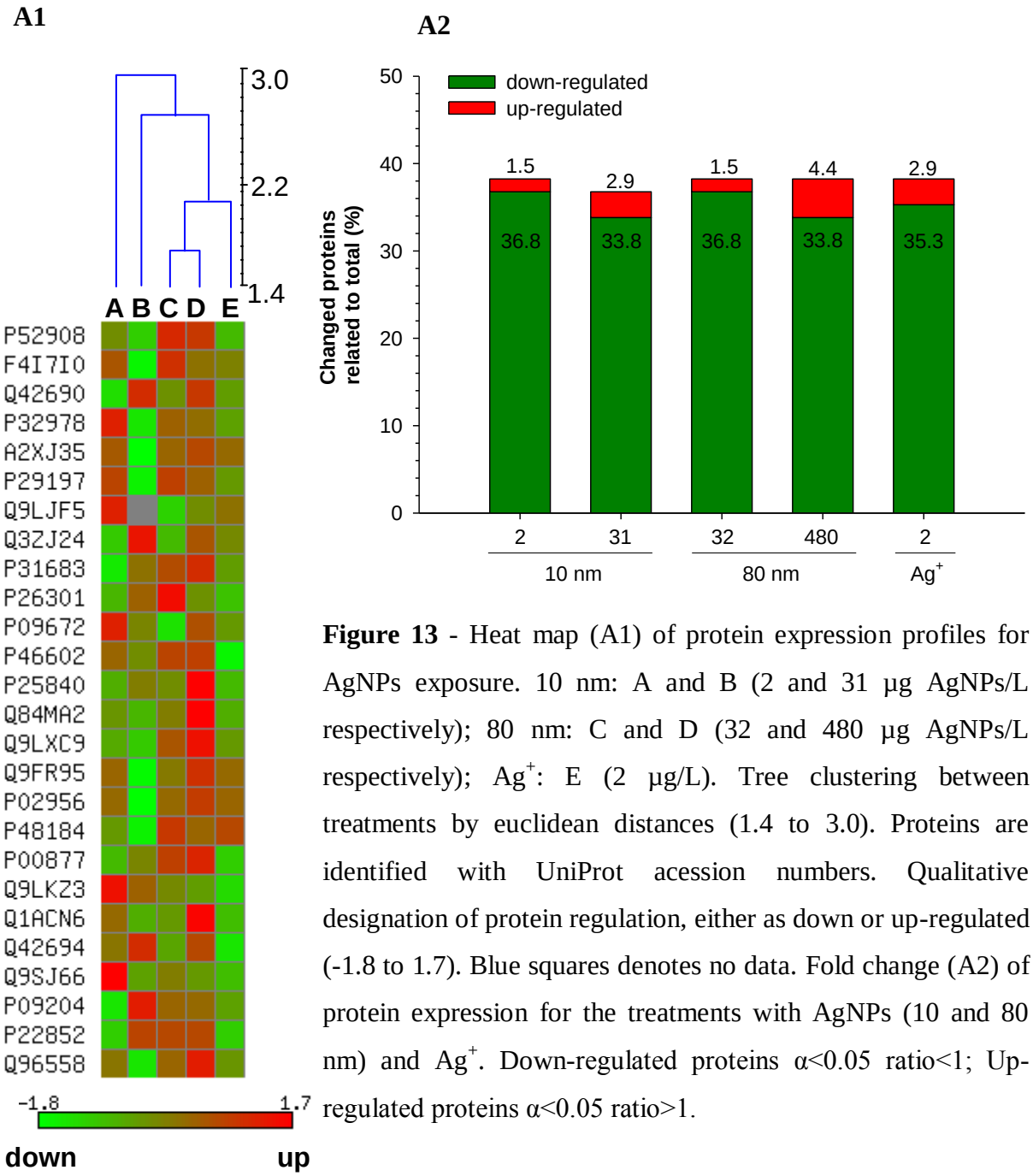

Figure 13 - Heat map (A1) of protein expression profiles for AgNPs exposure. 10 nm: A and B (2 and 31 µg AgNPs/L respectively); 80 nm: C and D (32 and 480 µg AgNPs/L respectively); Ag^+: E (2 µg/L). Tree clustering between treatments by euclidean distances (1.4 to 3.0). Proteins are identified with UniProt acession numbers. Qualitative designation of protein regulation, either as down or up-regulated (-1.8 to 1.7). Blue squares denotes no data. Fold change (A2) of protein expression for the treatments with AgNPs (10 and 80 nm) and Ag^+. Down-regulated proteins $\alpha<0.05$ ratio<1; Up-regulated proteins $\alpha<0.05$ ratio>1.

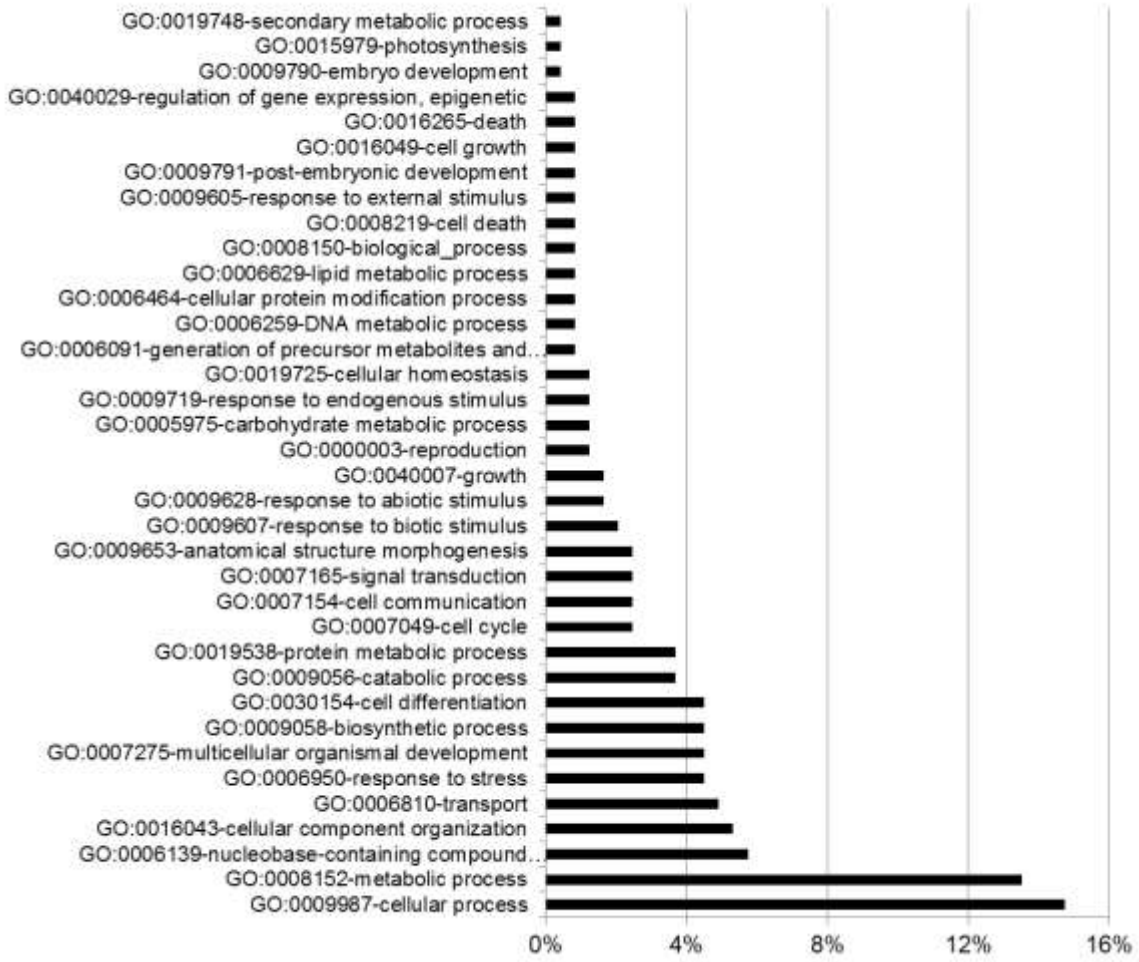

Figure 14 - Biological function as a percentage of the 26 regulated proteins (up or down) based on the AmiGO classification system. Performed using slim down associations with Plant GO slim.

Conclusions

The effect concentrations values of both AgNPs fall within the 1–10 mg/L so are classified as toxic to aquatic organisms (CEC, 1996). Moreover, the smaller AgNPs tested was 11-fold more toxic than the larger one for the *C. reinhardtii* grow endpoint. At sub-inhibitory concentrations oxidative stress biomarkers responded better to smaller AgNPs exposure, having early warnings at 5 µg/L for Chl *a+b* and GST. While for the larger, only the GST activity was affected at low concentration as 192 µg/L.

The exposure of *C. reinhardtii* to AgNPs had effects at protein level, giving early warnings at concentrations as low as 2 µg/L, fulfilling the lack of evidence on the toxicity effects of the 80 nm particles using biomarkers.

The integration of phenotypic endpoints, biomarkers and –omics technologies on toxicity assessment, will improve the understanding of the mode of action of AgNPs in aquatic systems. Furthermore, will also validate the use of the sub-cellular endpoints as early warning indicators and increase their ecological relevance.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Supplementary material

Table 2 - Protein abundance ratios determined by iTRAQ quantification compared with the several treatments. Ratios are presented as mean±SD.

		µg AgNPs /L								µg/L			
		10 nm				80 nm				Ag^+			
Acession #	**Name**	**2**		**32**		**31**		**480**		**2**			**Mass (Da)**
P52908	14-3-3-like protein	0.56 ± 1.75		0.50 ± 1.62		0.68 ± 1.75		0.65 ± 1.71		0.52 ± 1.67			35276
P52413	Acyl carrier protein 3	--	--	--	--	--	--	--	--	--	--		18122
P53498	Actin	--	--	--	--	--	--	--	--	--	--		47589
F4I7I0	Alanine aminotransferase 1	0.55 ± 1.31		0.40 ± 1.10		0.59 ± 1.29		0.52 ± 1.36		0.50 ± 1.39			69822
Q42690	Fructose-bisphosphate aldolase 1	0.55 ± 1.50		0.93 ± 1.49		0.70 ± 1.53		0.89 ± 1.47		0.67 ± 1.53			49781
Q9M3G7	Serine/threonine-protein kinase	--	--	--	--	--	--	--	--	--	--		507238
P32978	ATP synthase subunit beta	0.56 ± 1.59		0.44 ± 1.78		0.52 ± 1.64		0.51 ± 1.64		0.48 ± 1.68			58000
Q42687	ATP synthase delta chain	--	--	--	--	--	--	--	--	--	--		30977
Q8GY61	Transcription factor bHLH63	--	--	--	--	--	--	--	--	--	--		46034
Q6Z4U2	CRS2-associated factor 1	--	--	--	--	--	--	--	--	--	--		57388
A2XJ35	Chlorophyll a-b binding protein	0.37 ± 1.18		0.16 ± 1.11		0.35 ± 1.17		0.40 ± 1.28		0.34 ± 1.26			32128
Q8LGA1	Cyclin-D4-1	--	--	--	--	--	--	--	--	--	--		39865
Q3BAH8	Cytochrome c biogenesis protein	--	--	--	--	--	--	--	--	--	--		41676
Q8VY16	Plastid division protein CDP1	--	--	--	--	--	--	--	--	--	--		106584
P29197	Chaperonin CPN60	0.50 ± 1.03		0.38 ± 1.03		0.50 ± 1.02		0.47 ± 1.03		0.44 ± 1.19			77365
Q9LJF5	Double-stranded RNA-binding protein 3	0.07 ± 1.11		* ± *		0.01 ± 1.03		0.02 ± 1.02		0.03 ± 1.05			48262
B7E321	Double-stranded RNA-binding protein 5	--	--	--	--	--	--	--	--	--	--		46460
Q9SMH3	Dynein-1-alpha heavy chain	--	--	--	--	--	--	--	--	--	--		604343
O23755	Elongation factor 2	--	--	--	--	--	--	--	--	--	--		111686

Q9ZT91	Elongation factor Tu	--		--	--		--	--		--	--		--	--		--	58505	
Q3ZJ24	Elongation factor Tu	0.74	±	1.47	2.02	±	1.68	0.81	±	1.51	1.41	±	1.64	1.05	±	1.68	52994	
P26301	Enolase 1	0.45	±	1.23	0.59	±	1.27	0.64	±	1.30	0.69	±	1.25	0.53	±	1.26	49819	
P31683	Enolase	0.50	±	1.24	0.61	±	1.28	0.75	±	1.27	0.54	±	1.25	0.49	±	1.22	59289	
A2YVG8	Formin-like protein 9	--		--	--		--	--		--	--		--	--		--	116156	
Q9SV98	Putative F-box/kelch-repeat protein	--		--	--		--	--		--	--		--	--		--	49399	
P09672	Glyceraldehyde-3-phosphate dehydrogenase A	0.37	±	1.43	0.27	±	1.55	0.21	±	1.47	0.32	±	1.54	0.26	±	1.45	30258	
Q8VXQ8	Glyceraldehyde-3-phosphate dehydrogenase	--		--	--		--	--		--	--		--	--		--	41137	
P40280	Histone H2A	--		--	--		--	--		--	--		--	--		--	23414	
P46602	Homeobox-leucine zipper protein	0.60	±	1.16	0.56	±	1.24	0.63	±	1.35	0.63	±	1.31	0.48	±	1.27	40201	
P25840	Heat shock 70 kDa protein	0.90	±	1.59	0.97	±	1.58	0.94	±	1.55	1.17	±	1.61	0.88	±	1.60	85859	
Q08277	Heat shock protein 82	--		--	--		--	--		--	--		--	--		--	104655	
Q9LVA7	Chloride conductance regulatory protein	--		--	--		--	--		--	--		--	--		--	27412	
Q84MA2	Type I inositol 1,4,5-trisphosphate 5-phosphatase 1	0.70	±	1.22	0.65	±	1.23	0.73	±	1.25	0.94	±	1.27	0.66	±	1.25	75954	
Q9LXC9	Soluble inorganic pyrophosphatase 1	0.53	±	1.37	0.51	±	1.40	0.58	±	1.42	0.64	±	1.47	0.54	±	1.38	42181	
Q38796	Homeobox protein LUMINIDEPENDENS	--		--	--		--	--		--	--		--	--		--	123602	
O22042	Mitogen-activated protein kinase kinase kinase 3	--		--	--		--	--		--	--		--	--		--	84994	
A6H5E5	Maturase K	--		--	--		--	--		--	--		--	--		--	73297	
Q655R6	Molybdenum cofactor sulfurase	--		--	--		--	--		--	--		--	--		--	108205	
P19142	Phenylalanine ammonia-lyase class 2	--		--	--		--	--		--	--		--	--		--	90386	
Q9FR95	Arabidiol synthase	3.03	±	1.01	1.18	±	1.06	2.65	±	1.05	4.19	±	1.08	2.95	±	1.09	101402	
Q9FH87	Putative pentatricopeptide repeat-containing protein	--		--	--		--	--		--	--		--	--		--	91411	
Q6EW48	Photosystem I P700 chlorophyll a apoprotein A1	--		--	--		--	--		--	--		--	--		--	88902	
Q49CB2	Photosystem I P700 chlorophyll a apoprotein A2	--		--	--		--	--		--	--		--	--		--	88197	
P02956	Photosystem Q(B) protein	0.53	±	1.03	0.41	±	1.23	0.53	±	1.07	0.58	±	1.08	0.54	±	1.08	39244	
P48184	Photosystem II D2 protein	0.56	±	1.74	0.46	±	1.68	0.69	±	1.74	0.63	±	1.78	0.67	±	1.76	41099	
Q9LM20	Putative pumilio homolog 8, chloroplastic	--		--	--		--	--		--	--		--	--		--	64061	
P00877	Ribulose bisphosphate carboxylase large chain	0.61	±	1.32	0.67	±	1.31	0.76	±	1.33	0.80	±	1.28	0.59	±	1.32	59810	

Q9LKZ3	Retinoblastoma-related protein 1	0.07	±	1.27	0.03	±	1.96	0.02	±	2.32	0.01	±	2.90	0.01	±	4.36	130053
A7P514	Retinoblastoma-related protein	--		--	--		--	--		--	--		--	--		--	129592
Q93Z92	E3 ubiquitin-protein ligase	--		--	--		--	--		--	--		--	--		--	47363
Q1ACN6	DNA-directed RNA polymerase subunit beta	0.42	±	1.22	0.36	±	1.24	0.38	±	1.31	0.54	±	1.31	0.35	±	1.32	145573
Q42694	RuBisCO large subunit-binding protein subunit alpha	0.74	±	1.17	0.85	±	1.19	0.68	±	1.26	0.81	±	1.18	0.61	±	1.14	73689
Q9FKH1	Transcriptional regulator STERILE APETALA	--		--	--		--	--		--	--		--	--		--	49580
A5YVF1	Protein SUPPRESSOR OF GENE SILENCING 3	--		--	--		--	--		--	--		--	--		--	90127
Q9SJ66	Probable sucrose-phosphatase 2	0.17	±	1.13	0.06	±	1.22	0.08	±	1.12	0.07	±	1.15	0.05	±	1.10	59081
Q43847	Granule-bound starch synthase 2	--		--	--		--	--		--	--		--	--		--	98553
O65413	Sugar transport protein 12	--		--	--		--	--		--	--		--	--		--	62822
P09204	Tubulin alpha-1 chain	0.49	±	1.71	0.95	±	2.04	0.74	±	1.58	0.74	±	1.97	0.61	±	1.65	55639
P22852	Tubulin beta chain	0.50	±	1.02	0.61	±	1.03	0.61	±	1.05	0.61	±	1.14	0.50	±	1.14	54411
Q0WUI9	Probable alpha,alpha-trehalose-phosphate synthase	--		--	--		--	--		--	--		--	--		--	111796
P23400	Thioredoxin M-type	--		--	--		--	--		--	--		--	--		--	18438
Q96558	UDP-glucose 6-dehydrogenase 1	0.76	±	1.10	0.62	±	1.07	0.78	±	1.03	0.89	±	1.05	0.71	±	1.04	65076
Q6WWW4	E3 ubiquitin-protein ligase	--		--	--		--	--		--	--		--	--		--	231397
B8BDW1	Protein XAP5 CIRCADIAN TIMEKEEPER	--		--	--		--	--		--	--		--	--		--	49583
Q40082	Xylose isomerase	--		--	--		--	--		--	--		--	--		--	64531
Q5JNA1	B3 domain-containing protein	--		--	--		--	--		--	--		--	--		--	101165
Q9SN21	Putative BTB/POZ domain-containing protein	--		--	--		--	--		--	--		--	--		--	73519
Q9FLJ8	Probable receptor-like protein kinase	--		--	--		--	--		--	--		--	--		--	106318

Chapter III: Chronic effects of silver nanoparticles to *Daphnia magna*: aqueous and dietary exposure

Chronic effects of silver nanoparticles to *Daphnia magna*: aqueous and dietary exposure

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Abstract

Silver nanoparticles (AgNPs) are the most produced engineered nanomaterial, due to their high commercial value as active component of several products that render antimicrobial properties. However, is known that AgNPs can cause deleterious effects on non-target species, so it's high priority to evaluate their fate and effects on aquatic ecosystems.
Currently, there is no chronic toxicity studies on the exposure of *Daphnia* population's to AgNPs through diet. We performed a 21-day exposure of *Daphnia magna* to AgNPs via aqueous and dietary, and population performance. In the aqueous exposure, we found a significant delay on the release of the first brood at concentrations as low as 15 µg AgNPs/L, disruption of the initial reproduction in all AgNPs concentrations tested and a decrease on the total offspring production that followed a dose-response trend. On the dietary overview, we found that at 300 µg AgNPs/L of pre-exposed food a decrease in the *D. magna* survivorship occurred along the exposure, also a significant reduction on offspring production and a significant decrease in the final body weight. Taking in consideration the actual predicted environmental concentrations (PECs) we can infer that in a real-world event, most likely no impacts to *Daphnia* populations will occur.

Keywords: aquatic toxicology, silver nanoparticles, chronic toxicity, *Daphnia magna*

Introduction

Among the engineered nanomaterials (ENMs), silver nanoparticles (AgNPs) are the most produced (Wijnhoven et al., 2009; Gottschalk et al., 2010), partly due to their high commercial value as component of several products (textiles, cosmetics, food packaging materials, electronics and household products), as likewise their use by research institutions, either for environmental assessment or for medical applications is still increasing. (Fabrega et al., 2011).

The main applications of AgNPs are related to their antimicrobial activity, which is due to the release of ionic silver (Sotiriou and Pratsinis, 2010) though they are known to cause deleterious effects on non-target organisms as microalgae, crustaceans, fish and plants (Fabrega et al., 2011). Some authors admit that the mechanisms underlying AgNPs toxicity are derived from the bulk form, from their dissolution in aqueous suspensions (Liu and Hurt, 2010; Tejamaya et al., 2012; Silva et al., 2014). Others defend that AgNPs itself can render toxicity by making use of its intrinsic novel properties related with nano scale (Choi and Hu, 2008; Park et al., 2011). However it's not easy to attribute an effect to a specific cause, without a good experimental design, e.g. remove the effects related with bulk by the use of ligands (e.g. cysteine) (Navarro et al., 2008) or characterize the amount of their dissolution (e.g. dialysis) (Franklin et al., 2007).

Aquatic organisms, namely filter feeding invertebrates, such as *Daphnia*, might be exposed to contaminants from the water column as well as from food sources (Taylor et al., 1998). For silver nanoparticles, the effects through the water column (aqueous exposure) are reasonably known, but the effects from food sources (dietary exposure) remain quite unstudied. The exposure of organisms to contaminants via dietary can enhance phenomena such as bioaccumulation and biomagnification (Memmert, 1987; Zhu et al., 2010).

The main goal of this study was to evaluate the effects of uncoated AgNPs to *Daphnia magna* upon aqueous and dietary exposures. To attain the main goal four specific objectives were delineated (*i*) evaluate the effects of AgNPs on the growth rate of the green microalgae *Pseudokirchneriella subcapitata*; (*ii*) determine the acute toxicity and feeding rate of *D. magna* exposed to AgNPs; *(iii)* evaluate the effects of aqueous exposure of AgNPs in the survival, growth and reproduction of *D. magna; (iv)* evaluate the effects of dietary exposure of AgNPs in the survival, growth and reproduction of *D. magna*. The

assessment of the effects of both aqueous and dietary exposure of AgNPs to *D. magna* was based on 21 d chronic toxicity tests. For the dietary exposure, *D. magna* were fed with the algae *Pseudokirchneriella subcapitata* which have been previously exposed to AgNPs; both species are commonly used to illustrate a simple trophic chain. For the best of our knowledge, this study is the first evaluating the chronic effects of AgNPs to *Daphnia magna* through diet exposure.

Material and methods

1.1. AgNPs preparation and characterization

AgNPs were supplied by Sigma-Aldrich (St. Louis, MO, USA) with the highest degree of purity available, in form of powder, <100 nm in size and uncoated. Stock solutions were prepared in ultrapure water, sonicated at 42 kHz for 30 min and used immediately.

Particle size distribution was determined by dynamic light scattering (DLS) using a Zetasizer Nanoseries (Malvern Instruments, USA) with a 633-nm laser source and a detection angle of 173º (capable of detect particles from 1 nm to 10 mm). Measurements were conducted using a 1 ml sample deposited in a disposable polystyrene cuvette. Zeta potential was measured using a disposable electrophoretic flow-through cell with an internal volume of approximately 0.9 ml. These measurements were performed using 1 mg/l of AgNPs. To assess the effects of matrix type we performed these measurements in different matrices: ultrapure water (UPW), ASTM and MBL medium. To determine AgNPs changes over time we performed these measurements after 1, 24, 48 and 72 h.

SEM (Hitachi SU-70 operating at 4.0 kV) was used to visualize particle size, shape and aggregation state of AgNPs in ultrapure water suspensions (3.0 mg/L, pre-filtered with a 0.1 µm filter and carbon-coated). The size distribution of AgNPs was determined based on the measurement of particles diameter (n=160) using ImageJ (v1.47, NIH).

To measure the dissolution rate of AgNPs to ionic silver on both test media, we used a pre-treated tubing Spectra/Por 7 of 1 kD (MWCO) membrane dialysis (SpectrumLabs, USA), with 18 mm of flat width and 11.5 mm diameter. The tubing was cut in segments/sections with 9 cm length and rinsed in ultrapure water (UPW, Millipore Milli-Q Academic, USA) before use. We filled the cells (tubing segments) with 4 mL of test media and closed the

ends with Spectra/Por closures of 23 mm. Cells were then submerged in a polystyrene vial containing 200 mL of test media at 50 µg AgNPs /L. The only difference between the medium inside and outside the tubing segment/cell was that AgNPs were not present inside the tubing segment/cell. Measurements were performed in triplicate, 48 and 96 h after the start of exposure and total silver measurements were analyzed by ICP-MS.

1.2. Culture conditions

The freshwater unicellular green algae *P. subcapitata* were maintained in semi-continuous batch cultures of Woods Hole MBL medium (Stein and Hellebust, 1980) at 20 ± 1 ºC under continuous and uniform cool-white light and continuous aeration.

D. magna Straus (1820) (clone F, sensu Baird et al. (1991)) were cultured in synthetic ASTM hard water (ASTM, 1998) with a standard organic additive (Marinure seaweed extract, Glenside Organics Ltd.). Organisms were fed *P. subcapitata* (3×10^5 cell/ml) daily. Photoperiod was 16 h light: 8 h dark and temperature was set to 20 ± 1 ºC. The culture medium was renewed three times a week.

1.3. Bioassays

1.1.1. Algae tests

The growth inhibition test followed the OECD guideline 201 (OECD, 2006). Five AgNPs concentrations (0.15, 0.30, 0.60, 1.2 and 1.8 mg/l) plus a control were tested, using three replicates per treatment. The test was initiated with a density of 1.0×10^4 cells/ml in the log exponential growth phase and was carried out in 24 wells microplates at 24 ± 1 ºC with daily shaking and constant cool-white light (4000 lux). At the end of the test, the optical density at 440 nm (OD) was measured by spectrometry (Jenway 6505 UV/Visible spectrophotometer, UK) and converted to cell concentration (cells/ml) using the following equation (Cell concentration = -171.1 + Abs (440 nm) × $7.9.\times10^7$, r^2=0.97). Growth rates were determined as the logarithmic increase in biomass, measured as cell number (OECD, 2006).

With the goal of producing algae for the foodborne experiment with *D. magna*, algae were grown in the presence of AgNPs at concentrations below the 72h-EC_{20} (50, 100, 150, 200 and 300 µg/l) plus a control. The growth conditions were the same as those applied in

culture maintenance. After 7 days of exposure algae were harvested, centrifuged at 2500 rpm, 4 ºC, for 10 min, followed by three washing steps in ASTM medium.

1.1.2. *Daphnia* tests

Acute toxicity

Acute immobilization tests followed the OECD guideline 202 (OECD, 2004). Only newly released neonates (6-24h old) from the third to sixth clutches were used in bio-assays. Five replicates were used per treatment. Each replicate consisted of 5 organisms exposed to 50 ml of ASTM hard water (OECD, 2004) with the desired AgNPs concentration (0, 50, 100, 110, 115, 120, 200 and 250 µg/l). No food was provided; photoperiod, light intensity and temperature were as for cultures. After 48 h of exposure, the number of immobilized daphnids was recorded. Immobilization was defined as the inability to swim or move after 15 s of gentle agitation. An additional test was carried out with the supply of food (*P. subcapitata*, 3×10^5 cells/ml) using the following AgNPs concentrations: 75, 175, 350, 385 and 400 µg/l).

Feeding inhibition

Tests were carried out with fourth instar juveniles (about 4 days old) using three replicates per treatment. Each replicate consisted of three juveniles in a glass vial containing 50 ml of ASTM hard water, algae (*P. subcapitata*, 3×10^5 cells/ml), and the desired AgNPs concentration (0, 175, 350, 385, 400, 800 µg/l). Two blanks having no daphnids were added to the experimental setup. The vials were kept in the dark at 20 ± 1 ºC for 6 h. After the end of the exposure the vials were vigorously shaken and the absorbance was measured at 440 nm by spectrophotometry (Jenway 6505 UV/Visible spectrophotometer, UK). The absorbance was converted to cell concentration and these values were used to determine the feeding rates using the equation by Allen et al. (1995) with slight adaptations:

$$F=\frac{V\times(C_j - C_i)}{n\times(t_j - t_i)}$$

where F is the feeding rate (cells/animal × h); V is the volume of medium in the test vial (ml); C_i is the cell concentration at time i, and Cj is the cell concentration at time j; n is the

number of daphnids; t_i is the initial time of the exposure and t_j is the final time of the exposure.

Chronic toxicity

Two chronic reproduction tests were carried out: one to study the effects of aqueous AgNPs exposure and another one to study the effects of the dietary exposure of AgNPs to *D. magna*. The test by aqueous exposure was performed following the OECD guideline 211 (OECD, 2008) and were initiated with newly released neonates of *D. magna* (6-24 h old) from the third to sixth clutches. Fifteen replicates were used per treatment. Each replicate consisted of one individual exposed to 50 ml of ASTM hard water with the desired AgNPs concentration (0, 5, 15, 25, 35 and 55 µg/l). Daphnids were fed (*P. subcapitata*, 3×10^5 cells/ml) daily and medium was renewed every other day with freshly prepared AgNPs. During the media renewal, offspring as well as aborted eggs and neonates were counted and the moults were collected for posterior determination of daphnids body length (BL). BL (from head to the base of spine) was estimated based on the length of the first exopodite of the second antennae (AL) which was measured in the carapace released at the end of each instar. The following equation was used: ($BL = 10.98 \times AL - 0.55$ r^2=0.978, n=128, p<0.0001). AL was measured under a stereomicroscope (MS5, Leica Microsystems, Houston, TX, USA) with a built-in calibrated eyepiece micrometer.

Dietary exposure tests were performed under the same conditions as the aqueous exposure test, except that the algae used to feed the daphnids had been previously exposed to AgNPs (as described above). Both old and freshly prepared media were used to measure temperature, pH, conductivity and dissolved oxygen.

1.4. Statistical analysis

Sigma Plot 11.0 statistical software was used for statistical analyses. For normally distributed and homoscedastic data sets, checked by the Kolmogorov–Smirnov normality test and the Levene's test, respectively, a one-way ANOVA was used to detect the differences among treatments. Otherwise, the non-parametric Kruskal–Wallis test was used. If significant differences among treatments were found, the Dunnett or Dunn's tests

(for parametric or non-parametric tests, respectively) were used for multiple comparisons. The effect concentration (EC_{50}) values were calculated using a non-linear allosteric decay function in a spreadsheet built over Microsoft Excel. All statistical analyses were based on 0.05 significance level.

Results

AgNPs characterization

The size and stability of AgNPs in both toxicological media (ASTM and MBL) and in ultrapure water (UPW) as background reference were evaluated through hydrodynamic size and zeta potential for the most relevant exposure periods. Dissolution rate of AgNPs into Ag^{+} was verified by dialysis in both ASTM and MBL media for the most relevant exposure periods. The ionic strength and chloride concentration on both toxicological media were formulated by theoretical calculations. The results for these measurements are presented in Table 3.

The sizes of AgNPs increased significantly (p<0.001) with exposure time within matrices, with the exception of ASTM medium. Different types of matrix for the same exposure period differed significantly among each other (p<0.001) with the exception of ASTM and UPW at 24 h (Table 3).

The zeta potential (ζ-potential) varied between -12.8 mV in UPW and -26.7 mV in MBL, both for 1 h of exposure. Regarding particle stability this electrokinetic potential range is classified as incipient behavior (±10 to ±30) (Table 3).

The dissolution rate (%) of AgNPs in ASTM was 12 times higher than in MBL. The calculated ionic strength was 3.6 times higher in ASTM than in MBL. In opposition, the chloride concentration was 10 times lower in ASTM than in MBL (Table 3).

Table 3 - Hydrodynamic size, zeta potential (ζ-potential), dissolution rate, theoretical ionic strength and theoretical chloride (Cl^-) concentration in the matrices used for testing AgNPs toxicity - ASTM, MBL and ultrapure water as reference (UPW) for several exposure periods.

Matrix	Exposure (h)	Size (nm)	ζ-potential (mV)	Dissolution rate (%)	Ionic strength (mmol/L)	$[Cl^-]$ (mmol/L)
UPW	1	57.0±0.6	-12.8±5.5	-	0	0
	24	79.4±0.7	-26.5±4.5			
ASTM	1	78.3±2.6	-13.9±6.9	*4.80±1.0 ^{48h}	44.4	0.23
	24	80.0±0.7	-14.9±4.5			
MBL	1	119.3±0.2	-26.7±5.6	*0.40±0.2 ^{96h}	12.2	2.35
	24	104.1±2.0	-24.6±3.9			
	48	157.2±0.5	-22.0±5.5			
	72	322.6±1.5	-24.3±4.9			

*Pereira *et al*. unpublished data

SEM was used to visualize shape, size and aggregation state of AgNPs. Silver presence in samples was evaluated qualitatively with energy-dispersive X-ray spectroscopy (EDX) (not shown). The visual inspection of SEM pictures shows that the majority of AgNPs have quasi-spherical shape and tendency for clustering and consequently forming aggregates of more than 500 nm in length (Figure 15A). The diameter of individual nanoparticles counts based on SEM was 89.9±26.7 nm and their distribution was lightly positively skewed (n=160, Figure 15B).

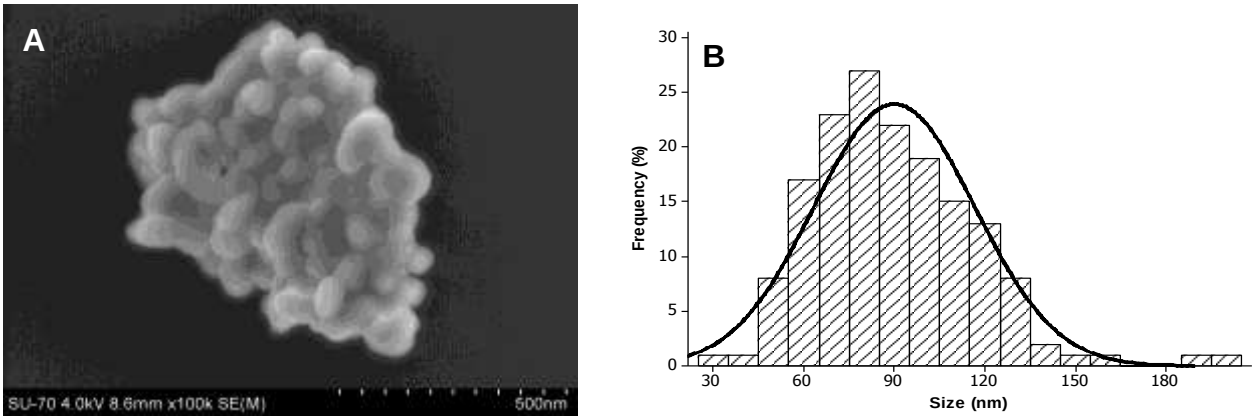

Figure 15 - Visual characterization of AgNPs: A) SEM picture of a AgNPs cluster formed after 24 h of exposure in ultrapure water; B) size distribution of AgNPs.

Short-term effects of AgNPs to *P. subcapitata* and *D. magna*

The effects of AgNPs to *P. subcapitata* and *D. magna* are presented in Table 4. The 72h-EC_{50} for the algae was 0.50 mg/L (Table 4), which is the highest EC_{50} value determined. The acute toxicity of AgNPs to *D. magna* was 3.4-fold higher in the absence of food than in the presence of food (Table 4). The toxicity of AgNPs in the presence of algae is very similar for both endpoints (immobilization and feeding rate). *D. magna* was the most sensitive tested specie regarding short-term toxicity to AgNPs (Table 4).

Table 4 - Summary of the short-term toxicity of AgNPs to *P. subcapitata* (growth inhibition) and *D. magna* (feeding rate and immobilization) in the absence (-) or in the presence (+) of food (algae). EC_{20} and EC_{50}±CL (confidence limits at 95%) are given in mg/l. (r^2 represents the coefficient of determination).

Test species	Endpoint	Food	Exposure	Age	EC_{20} ±C.L.	EC_{50} ±C.L.	*p* (r^2)	*p* (F)
P. subcapitata	Growth	-	72 h	log-phase	0.30±0.03	0.50±0.03	0.94	
D. magna	Feeding rate	+	6 h	4 d	0.33±0.04	0.35±0.01	0.89	<0.05
	Immobilization	-	48 h	<24 h	0.10±0.01	0.11±2.51E-03	0.88	
		+			0.38±0.02	0.39±2.85E-03	0.93	

Effects of AgNPs to *D. magna* through aqueous exposure

In this experiment daphnids were exposed through medium to different concentrations of AgNPs, and the chronic effects on life history traits were assessed (Figure 16).

The main effects of AgNPs to *D. magna* through aqueous exposure are depicted in Figure 16. The effects on survival were highly pronounced at high concentrations, with 80 and 73% mortality, respectively for 35 and 55 µg/L (Figure 16A). Reproduction, represented by the total offspring production per *Daphnia*, followed a dose-response curve (Figure 16B) with an EC_{50} value of 21.3±3.8 µg AgNPs/L. The release of the 1st brood was significantly delayed in the AgNPs concentrations on the range of 15 to 55 µg/L (Figure 16C). For the lower AgNPs concentrations no effect was found on both offspring production and time to 1st brood (Figure 16, B and C). The 1st brood of *D. magna* was severely affected by the initial AgNPs exposure as verified by the high number of aborted eggs at instar #5 (Figure 16D).

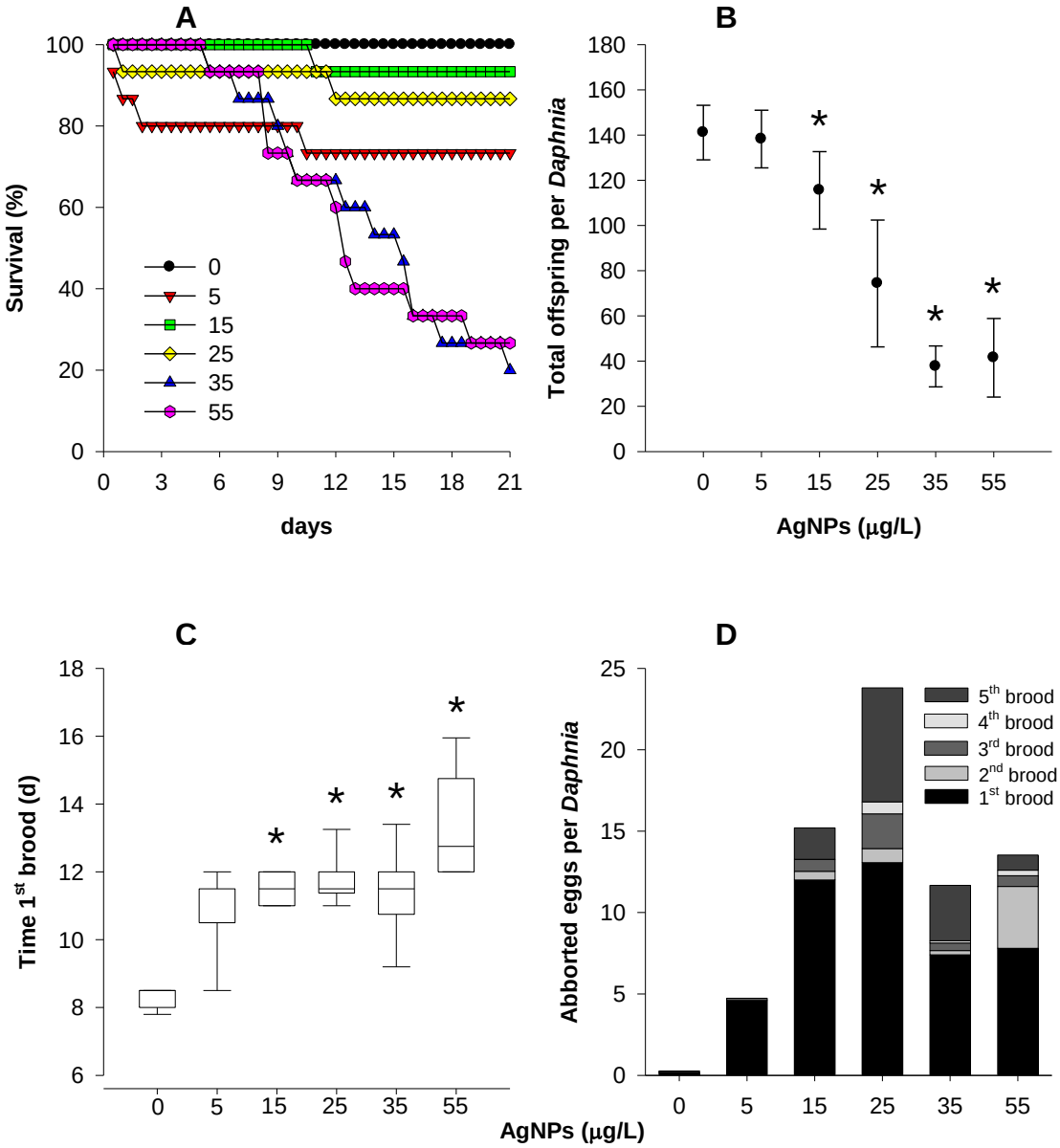

Figure 16 - Effects of AgNPs to *D. magna*, through aqueous exposure, after a 21 days period, on survival (A) total offspring per *Daphnia* (B), time to 1st brood (C) and aborted eggs per *Daphnia* at each instar stage (instar #5 is equivalent to the 1st brood) (D).Time to 1st brood represents the time elapsed between the start of the test and the release of neonates from the brood pouch to the external medium. Comparisons were made with control group (0) and data is reported as mean ± SD (total offspring per *Daphnia*, Tukey test) and median with 5th/95th percentiles (time to 1st brood, Dunn's method) **p* <0.05. In B calculated EC_{50} for reproduction, 21.3 with a 95% C.L. of 17.5-25.2 µg AgNPs/L.

Effects of AgNPs to *D. magna* through diet exposure

In this experiment daphnids were fed with algae previously exposed to different concentrations of AgNPs, and the chronic effects on life history traits were assessed (Figure 17).

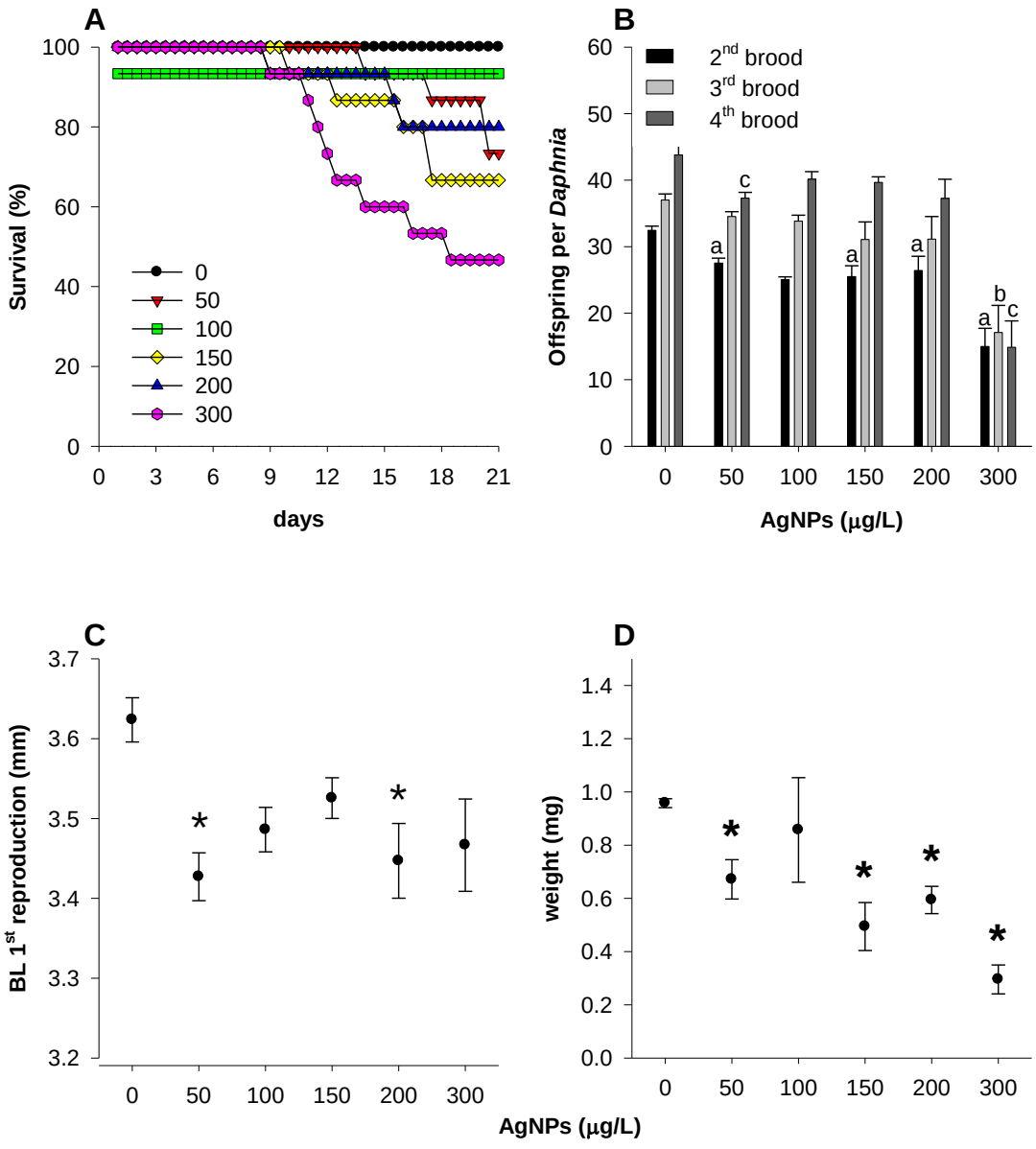

Figure 17 - Effects of AgNPs to *D. magna*, through diet exposure, after a 21 days period, on survival (A), offspring production at each instar (B), body length (BL) of adults at the start of the 1st reproduction (C), final weight of adults (D). Comparisons were made with control group (0). Data are reported as mean ± SE Dunn's method) [a, b, c,] $*p < 0.05$.

The effects on the survival were moderately pronounced at higher concentrations, reaching 50% of mortality in the 300 µg/L treatment (Figure 17A). Overall, the offspring production was more affected by the dietary exposure of AgNPs at the instar #6 (equivalent to the 2nd brood), with significantly differences from the control at the 50, 150, 200 and 300 µg/L (Figure 17B). Although no significantly differences were found in the number of neonates at the 1st reproduction, significantly differences were found in the body length of adults at the start of the 1st reproduction, for the 50 and 200 µg/L treatments (Figure 17C). The final weight of *D. magna* adults was statistically significant for all treatments with the exception of 100 µg/L (Figure 17D).

Discussion

In this work we aimed to assess the chronic toxicity of AgNPs both from the aqueous phase and from the diet to the freshwater micro-crustacean *D. magna*. We selected uncoated AgNPs with size less than 100 nm since these characteristics better represent the physico-chemical properties, namely surface modifications and size polydispersity, of particles that could be found in a real-word situation, such as an event of effluent release into aquatic systems.

The observed effect concentration of AgNPs to the microalgae *P. subcapitata* (72h EC_{50}= 500 µg/L) falls within the range of values reported in previous studies. Indeed, the effect concentrations of AgNPs to freshwater microalgae reported in literature is highly variable, ranging from 5 µg/l (Table 3) to 20 mg/l (Miao et al., 2010). This high variation within AgNPs and algae is due to the several combinations that can be assigned on the AgNPs features e.g. size, coating type, surface charge and functional groups (Fabrega et al., 2011). For instance, the toxicity data to *P. subcapitata* available in the literature is based on the size range from 3 to 30 nm (Table 5). Thus, the higher effect concentration obtained in our study compared to the study of (McLaughlin and Bonzongo, 2012) might be due to the higher size of the AgNPs used in our study (Table 3).

The feeding activity of *D. magna* is affected in 50% at 350 µg/ AgNPs/L, being very similar to the acute toxicity of *D. magna* neonates in the presence of microalgae (Table 2). The sedimentation rate of microalgae can increase when they adsorb AgNPs, thus becoming less available for daphnids (Ribeiro et al., 2014). Otherwise AgNPs can

accumulate in daphnids guts by the dietary intake, causing adverse issues on AgNPs excretion (Zhao and Wang, 2011).

On the other hand, toxicity data for the immobilization of *D. magna* is abundant both for different sizes and coating types of AgNPs. However, for clarity, only data relative to sizes between 60 and 300 nm was selected. The effect concentration for immobilization of *D. magna* (neonates) found in this study was 110 and 390 µg/L, respectively in the absence and presence of algae. The higher toxicity in the absence of algae agrees with previous results (Ribeiro et al., 2014). This difference, which is about 3.5-fold, is probably due to the adherence of AgNPs to the surface of algae and, therefore, its removal from the water column due to sedimentation of the algae.

Table 5 - Short-term toxicity data of AgNPs to both tested species *P. subcapitata* and *D. magna* found in the literature (selected taking in account coating type and size, for a proper comparison).

Species	Endpoint	Life stage	Exposure	$EC(L)_{50}$ (µg/l)	Size (nm)	Method	Coating	Reference
P. subcapitata	Chlorophyll *a*	log-phase	96 h	4.61	25.4	(TEM)	uncoated	(McLaughlin and Bonzongo, 2012)
	Growth			190.00	20-30	(provider)	citrate	(Griffitt et al., 2008)
			72 h	33.79	3–8	(TEM)	paraffin	(Ribeiro et al., 2014)
D. magna	Immobilization	neonates	24 h	531.50	<100	(provider)	uncoated	(Jo et al., 2012)
				1404.60	<150	(provider)	uncoated	
			48 h	0.75	60-100	(provider)	uncoated	(Lee et al., 2012)
				1.40	300	(provider)	uncoated	(Kim et al., 2011)
		adults		28.70	<100	(TEM)	lactate	(Zhao and Wang, 2012)

D. magna was most sensitive to AgNPs than the microalgae *P. subcapitata* (this study, Table 3). The exposure of *D. magna* to AgNPs through the water had a high impact on the total offspring production; the response to this endpoint followed a dose-response curve, reaching a 50% effect at 21 µg AgNPs/L (Figure 16B). This value is the second lowest EC_{50} reported for the reproduction performance in daphnids, after the value of 1.0 µg AgNPs/L reported by (Ribeiro et al., 2014). Furthermore, a significant delay on the release of the first brood (time to 1st brood) at concentrations as low as 15 µg AgNPs/L was found (Figure 16C). Additionally a high number of aborted eggs was found at the 1st reproduction stage for all AgNPs treatments (instar #5, Figure 16D). Regarding population dynamics, the population growth rate was strongly constrained (data not shown) at 35 and 55 µg AgNPs/L, where the population was in decline due to reduced survivorship and failure to reproduce. Comparatively to the aqueous exposure; the dietary exposure of daphnids had no such severe effects. Indeed, for the dietary exposure the most pronounced effects were observed for daphnids fed with algae grown at 300 µg AgNPs/L. These daphnids showed a remarkable decrease in the survivorship pattern along the 21 days exposure period, a significant reduction in the offspring production on the 2nd, 3rd and 4th moments of reproduction (instar #6, 7 and 8, respectively) and a significant decrease in body weight (Figure 17A, B, C and D).

Concerning nanoparticles characterization, some studies of (eco)toxicology perform it in e.g. DI or ultrapure water and extrapolate to more complex media. Such characterization does not reflect the properties of nanoparticles in the medium which was used to test for biological activity. For this reason we characterized AgNPs in MBL and ASTM media and observe their behavior on appropriate exposure times. Considering the size values obtained in DLS at the exposure time of 24 h (Table 3), our results are within the nominal size provided by the manufacture, in ultrapure water and in the test media. These results contradict some previous results. For instance, (Zhao and Wang, 2011) exposed the same type of AgNPs to complex media, and obtained higher average sizes (DLS measurements). This can be explained by the fact that many authors use the stock solution or higher AgNPs concentrations to characterize particles, (Allen et al., 2010; Kim et al., 2011) resulting in a faster AgNPs dissolution to Ag^{+}, which enhances the agglomeration and in consequence the aggregation state of particles (Jo et al., 2012).

The current need to dissociate nanoparticles effects from its size-related and particle solubility is fully recognized by the Organization for Economic Co-operation and Development in their final report of the prioritization of important issues on risk assessment of manufactured nanomaterials (MNs), where they ranked the physico-chemical properties with a top score as a subject of high to very high relevance to risk evaluation of the environment and human health (OECD, 2013). In order to distinguish these effects and characterize metal-based nanoparticles, several authors have been using fractionation techniques such as ultrafiltration (Kennedy et al., 2010; El Badawy et al., 2011), ultracentrifugation (Kennedy et al., 2010), Field flow fractionation (FFF) (Cumberland and Lead, 2009; Kennedy et al., 2010), membrane dialysis (Burchardt et al., 2012) and ligands of e.g. Ag^{+} as cysteine (Navarro et al., 2008; Yin et al., 2011) to remove or attenuate the effects associated with the bulk silver. The presence of a coating serves not only to stabilize the particle, but also to mediate their dissolution (Reidy et al., 2013). And this issue has been addressed in several studies (Ma et al., 2012; Nguyen et al., 2013; Silva et al., 2014). As likely other compounds, coated materials are also subject to chemical and physical deterioration, thus is expected a decay of the coating with time (Tejamaya et al., 2012), and consequently freeing/loosing from the particle (Reidy et al., 2013). In this study, the presence of chelating agents in MBL medium (i.e EDTA) can explain partially the low dissolution rate and higher stability comparatively to ASTM medium (Table 3).

The lack of quantitative measurements of AgNPs in the aquatic compartment is a major constraint for a reliable environmental risk assessment. However, predicted environmental concentrations (PECs) taking in consideration the life-cycle of products exist, regarding Europe surface waters and sewage treatment facilities, being the values between 0.59-2.2 ng/L and 32.9-111 ng/L, respectively (Gottschalk et al., 2009). Taking in consideration this predicted values and the results of acute and chronic exposures we can infer that in a real-world event, most likely no impacts to *Daphnia* populations would occur. However, no definitive conclusion should be taken, because of the absence of studies addressing multi-generational exposures of AgNPs to *D. magna* as well the effects of abiotic factors such as temperature and UV-light. Further research also should be made on assessing the possible impacts on the population distribution structure (e.g neonates, juveniles and adults).

Acknowledgments

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CHAPTER IV: Silver nanoparticles toxicity: species sensitivity distributions and general trends

Silver nanoparticles toxicity: species sensitivity distributions and general trends

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Abstract

Despite the increasing knowledge of the toxicity of silver nanoparticles (AgNPs) at the individual level, little is known about their effects at the community level. The main goal of this study was to extrapolate AgNPs toxicity to a broader range of aquatic species, using a species sensitivity distribution (SSD) approach. Following this approach the hazardous concentrations affecting 5% of the species in a community (HC5) and the predicted no effect concentration (PNEC) will also be determined. In addition, AgNPs toxicity to aquatic organisms will be described as a function of several properties, including the surface-area-to-volume-ratio.

The SSD showed that the most and least sensitive species were, respectively, the macroalgae *Chara vulgaris* and the crustacean *Thamnocephalus platyurus*. However, in general, the most and least sensitive groups are, respectively, crustaceans and fish. The predicted toxicity for the 5 % most sensitive organisms (HC5) is 0.062 mg/L and the derived PNEC varies between 0.012 and 0.062 mg AgNPs/L. Concerning the SSD in function of the AgNPs surface-area-to-volume-ratio, the HC5 is 3.79×10^{13} nm^2 /L and the derived PNEC varies between 7.59 $\times 10^{12}$ and 3.79 $\times 10^{13}$ nm^2 /L. The most and least sensitive groups are, respectively, plants and algae.

AgNPs toxicity is positively correlated with surface-area-to-volume-ratio, with a coefficient of determination 0.856. In opposition, AgNPs toxicity is weakly correlated with the size. This study showed that no toxicity is expected to algae, plants, crustaceans and fish, since the PEC values are lower than the PNEC values. However, further studies should be carried out before excluding potential toxicity of AgNPs to aquatic organisms in freshwaters.

Keywords: silver nanoparticles, community, aquatic organisms

Introduction

The modern society is dependent on nanotechnology and in the resulting nanoproducts, which is related to the fast growth in the development of new nanotechnologically-enabled particles, materials and products that has been observed in the past decades. Among the wide variety of nanotechnologically-enabled particles, silver nanoparticles (AgNPs) are the most produced and, moreover, their production is expected to increase in the future (Wijnhoven et al., 2009; Gottschalk et al., 2010). AgNPs are used in a wide variety of products, namely textiles, cosmetics, food packaging materials, electronics and household products, as well as in medical applications (Fabrega et al., 2011). Their wide application is due to the antibacterial, antifungal and anti-inflammatory properties of silver (Wijnhoven et al., 2009), which is released from AgNPs. Previous studies showed that silver can be released from consumer products containing AgNPs, reaching the aquatic environment. This, allied to the high production of AgNPs and to the high toxicity to aquatic organisms raises concern about their effects on aquatic ecosystems. AgNPs are 'very toxic' to aquatic organisms (Bondarenko et al., 2013), namely microalgae, crustaceans, fish and plants (Fabrega et al., 2011).

However, AgNPs toxicity to a certain species may vary widely. AgNPs toxicity is dependent on their intrinsic properties (size, shape, chemical composition of the capping agents, charge, surface structure and area, solubility, and aggregation state), as well as on the chemical properties of the exposure medium (pH, ionic strength and composition, organic matter and temperature) (Navarro et al., 2008).

Despite the increasing number of studies focusing on AgNPs toxicity to species at the individual level, little is known about their effects at the community level. AgNPs toxicity can be extrapolated to a broader range of species, using a species sensitivity distribution approach (SSD), which requires assembling of single-species toxicity. SSDs are one of the recommended approaches for ecological risk assessment and are used to predict hazardous concentrations (HC) affecting a certain percentage of species in a community. Commonly, this approach is used to determine HC5, the Hazard Concentration at 5 % level, i.e., the concentration that should protect 95 % of species. Following this approach, the predicted no effect concentration (PNEC) is also determined. Thus, this study aims to extrapolate AgNPs toxicity to a broader range of aquatic species, using a SSD approach. Following

this approach, the HC5 and PNEC will also be determined. In addition, AgNPs toxicity to aquatic organisms will be described as a function of several properties, including the surface-area-to-volume-ratio. To the best of our knowledge, this is the first study addressing the ecological risk assessment of AgNPs based on SSDs.

Material and methods

2.1 Gathering of data

All data used to derive the cumulative distribution functions (CDF) were collected from published literature (Table 1) and also from unpublished works of our research group. Concerning the latter, we used data on toxicological studies for microalgae (*Chlorella vulgaris* and *Pseudokirchneriella subcapitata*), crustaceans (*Daphnia magna*) and the aquatic macrophyte *Lemna minor*.

Special attention was taken to uniform L(E)C50 or minimum inhibitory concentrations (MICs) (not used in this study) values related with organisms age, lifestage, exposure time and ecological endpoint.

2.2. SSDs construction

Multiple toxicity data for the same species were summarized as geometric means. Data was adjusted to a log-probit distribution and the HC5 determined. The SSD plot was generated using the EPA spreadsheet (SSD Generator V1, downloaded from http://www.epa.gov/caddis/da_ software_ssdmacro.html). The PNEC value was calculated as the derived HC5 divided by a factor 1–5 (Posthuma et al., 2010).

Two SSDs were constructed. Following the common approach, a SSD representing the proportion of affected species (y-axis) as a function of the AgNPs concentration (x-axis) at the species level was developed. Following another approach, a SSD representing the percentage of affected species as a function of AgNPs surface-area-to-volume-ratio at the species level was also developed. In addition, for each SSD, data was gathered forming groups of organisms: algae, crustaceans, fish and plants. This allowed a better comparison of the sensitivity to AgNPs among groups of organisms.

2.3. Calculation of the surface-area-to-volume-ratio of AgNPs (only for quasi-spherical AgNPs)

In order to calculate the surface-area-to-volume-ratio (nm^2 /L) of AgNPs, we followed:

(1) Calculate the surface area per particle (A, in nm^2) of each AgNPs size class, through $A = 4\pi r^2$, where r=radius;

Multiplying the density of silver (10.49 g/cm3) by the average volume of an NP to determine the mass of an individual NP in each size class;

(2) Calculate the mass of each nanoparticle (g/ particle) by multiplying the silver density (10.49 g/ cm^3) with the volume of each particle (nm^3 /particle);

(3) Calculate the number of particles per volume (L) by the division of the AgNPs concentration by the mass of each particle;

(4) Multiplying the surface area of each particle by the number of particles per volume, we achieved to the surface-area-to-volume-ratio of AgNPs (Bowman et al., 2012).

Results and Discussion

SSDs for AgNPs concentration

Figure 18 shows the SSD representing the proportion of affected species as a function of the AgNPs concentration at the species level. The most sensitive species was the macroalgae *Chara vulgaris*, whereas the least sensitive was the crustacean *Thamnocephalus platyurus*.

The predicted toxicity for the 5 % most sensitive organisms (HC5) is 0.062 mg/L (0.021-0.185; r^2=0.951; N=16), lower and upper limits, respectively. The derived PNEC (Predicted No Effect Concentration) varies between 0.012 and 0.062 mg AgNPs /L. This range is above AgNPs concentrations predicted in the aquatic environment. Indeed the predicted environmental concentration (PEC) of AgNPs in surface waters in Europe and U.S are, respectively 0.764 and 0.116 ng L^{-1} (Gottschalk et al., 2009). The PEC is higher for the sewage treatment plant effluents: 32.9-111 ng L^{-1} and 16.4-74.7 ng L^{-1}, respectively

for Europe and U.S (Gottschalk et al., 2009). Thus, no toxicity is expected to algae, plants, crustaceans and fish, since the PEC values are lower than the PNEC values.

The distribution of sensitivities for organism groups (algae, crustaceans, fish and plants) is depicted in Figure 19. This curve shows that the most and least sensitive groups are, respectively, crustaceans and fish. Such distribution agrees with previous studies (Bondarenko et al., 2013), emphasizing the fact that crustaceans are the most sensitive group to AgNPs.

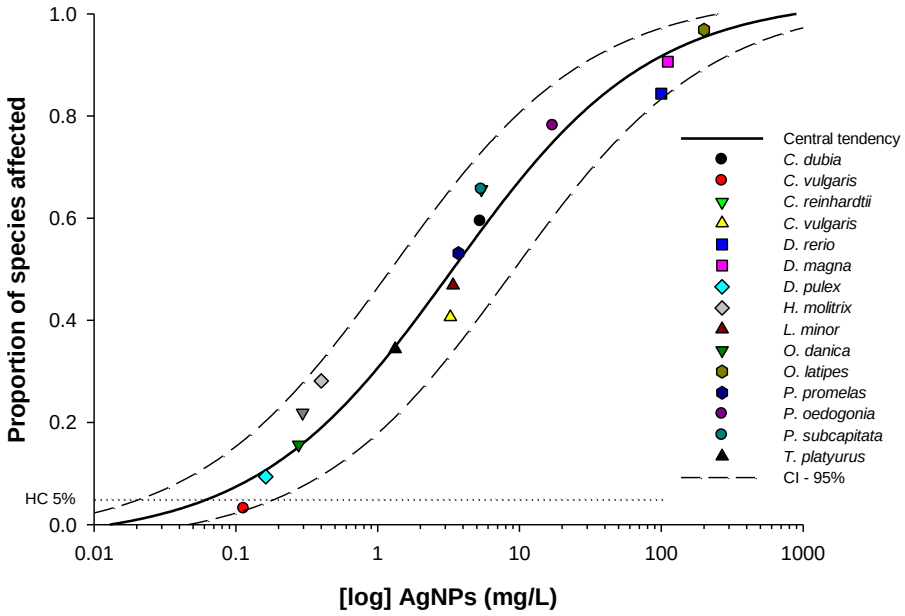

Figure 18 - SSD of AgNPs toxicity, expressed as concentration (mg/L) at the species level

This high sensitivity of crustaceans agrees with the high sensitivity of crustaceans to the silver ion (Bondarenko et al., 2013). In an acute pollution event, crustaceans (the most sensitive group) could be adversely affected, decreasing the feeding pressure on algae with potential consequences for ecosystem functioning by decoupling of trophic relationships.

The predicted toxicity for the 5 % most sensitive organisms (HC5) is 0.026 mg/L (0.001-1.313; r^2=0.910; N=4), lower and upper limit, respectively. The derived PNEC (Predicted No Effect Concentration) varies between 5.2 µg/L and 0.026 mg/L of AgNPs.

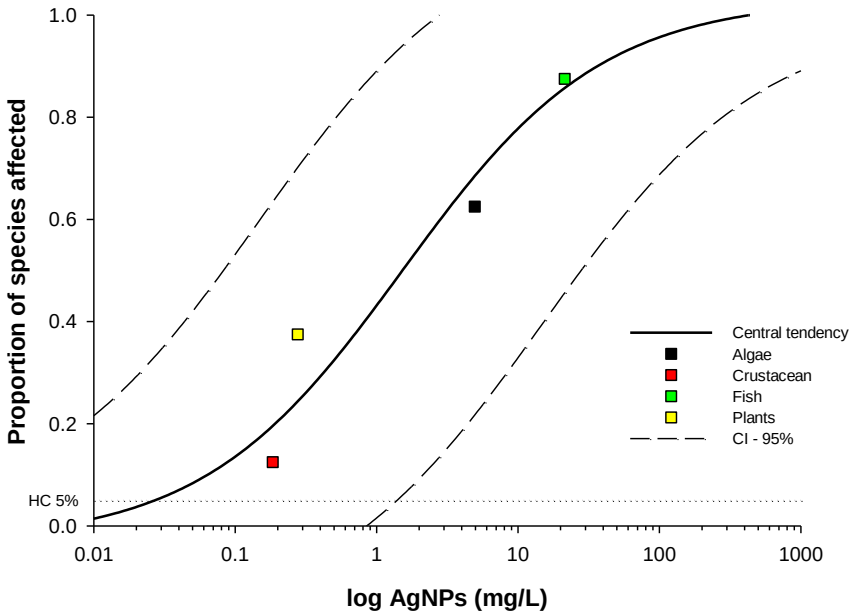

Figure 19 - Distribution of AgNPs toxicity, expressed as concentration (mg/L), to groups of organisms: algae, crustaceans, fish and plants

1.2. SSDs for AgNPs surface-area-to-volume-ratio

The SSD for the AgNPs surface-area-to-volume-ratio at the species level is depicted in Figure 20. The predicted toxicity for the 5 % most sensitive organisms (HC5) is 3.793E+13 nm^2 /L (5.649E+12 -2.877E+14; r^2=0.912; N=16), lower and upper limit, respectively. The derived PNEC (Predicted No Effect Concentration) varies between 7.586E+12 and 3.793E+13 nm^2 /L.

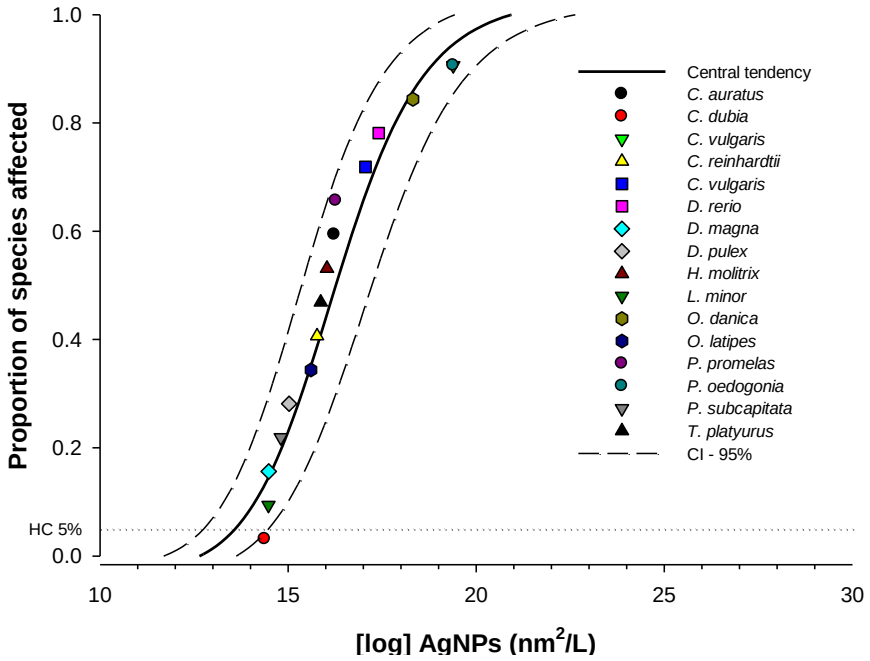

Figure 20 - SSD of AgNPs toxicity, expressed as surface-area-to-volume-ratio (nm^2/L) at the species level

Similarly, the distribution of sensitivities concerning groups of organisms is depicted in Figure 21. This Figure shows that, despite the narrow range of AgNPs surface-area-to-volume-ratio, the most and least sensitive groups are, respectively, plants and algae.

The predicted toxicity for the 5 % most sensitive organisms (HC5) is 2.695E+13 nm^2 /L (7.831E+10 - 3.614E+16; r^2=0.810; N=4), lower and upper limit, respectively. The derived PNEC (Predicted No Effect Concentration) varies between 5.390E+12 and 2.695E+13 nm^2 /L.

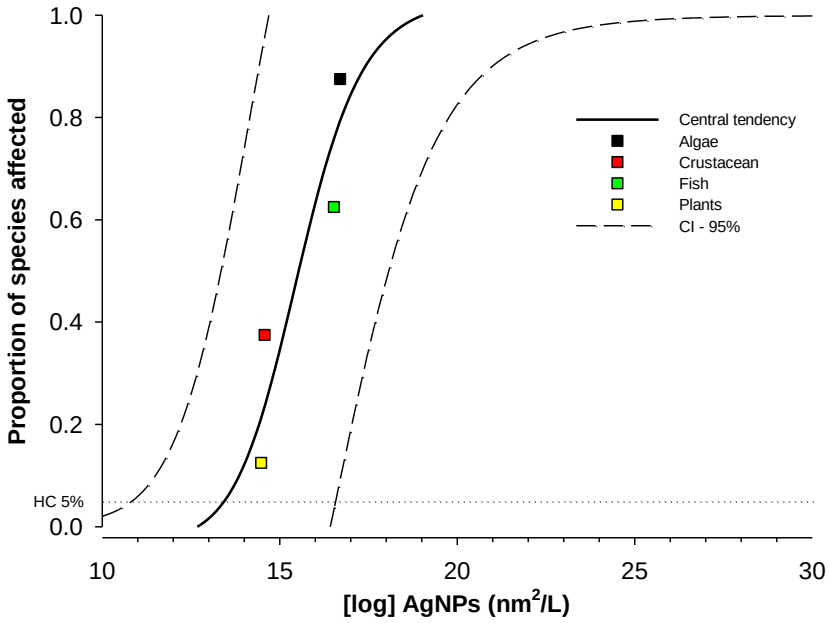

Figure 21 - Distribution of AgNPs toxicity, expressed as surface-area-to-volume-ratio (nm^2/L), to groups of organisms: algae, crustaceans, fish and plants

General trends in AgNPs toxicity

The toxicity of AgNPs to aquatic has been shown to be dependent on their size. However, there is a weak correlation between the EC_{50} values of AgNPs and the size of AgNPs, as illustrated in Figure 22. On the other hand, there is a high correlation (r^2=0.856) between the effect concentration values (EC_{50}) of AgNPs and the surface-to-area-volume-ratio. Both variables are positively correlated, as shown in Figure 23.

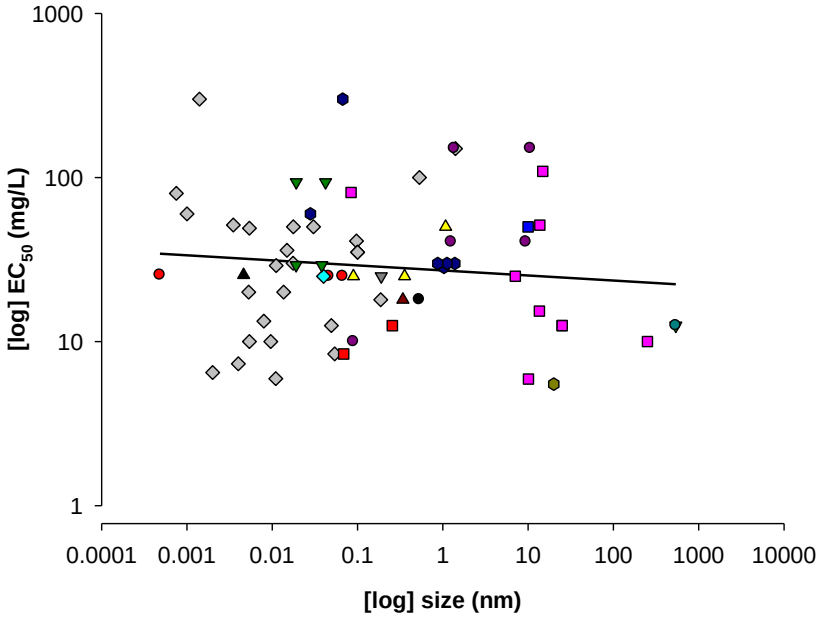

Figure 22 - Relationship between the effect concentration (EC_{50}) of AgNPs to aquatic organisms and the size (r^2=0.01).

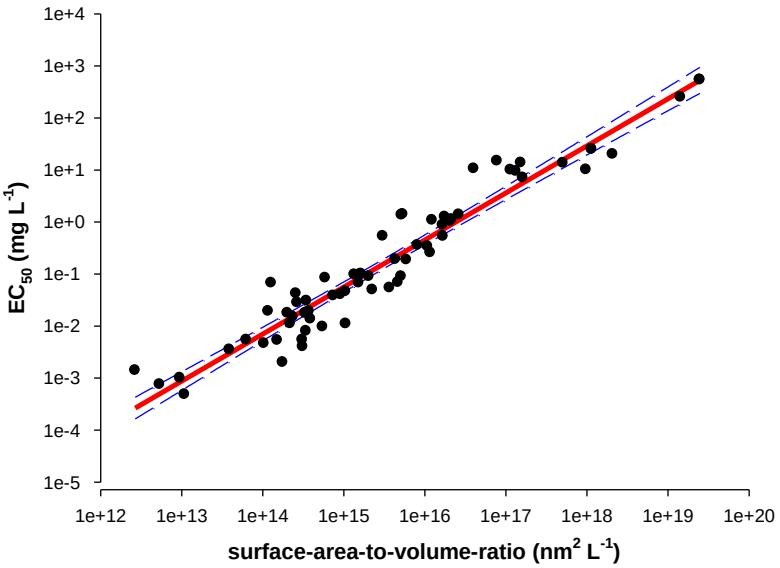

Figure 23 - Relationship between the effect concentration (EC_{50}) of AgNPs to aquatic organisms and the surface-area-to-volume-ratio (SAV ratio). The equation describing the data is: EC_{50} = 0.405*ln(SAV ratio) - 15.205; r^2=0.856.

Given this, we suggest that the variable surface-area-to-volume-ratio might describe the toxicity of AgNPs better than the size. This might also be valid for other nanoparticles.

Conclusions

This study showed that no toxicity is expected to algae, plants, crustaceans and fish, since the PEC values are lower than the PNEC values. However, aquatic organisms are not only exposed to AgNPs but also to the silver ion (Ag^+), which is recognized as the most toxic silver form to aquatic organisms (Hogstrand and Wood, 1998). Moreover, organisms are exposed to AgNPs not only through the water but also through the diet. Moreover, AgNPs accumulate in the sediment, and might be released to the water column under certain environmental conditions. Thus, toxicity of AgNPs to aquatic organisms should not be excluded before further research. In addition, the surface-area-to-volume ratio was shown to describe AgNPs toxicity better than the size, and we recommend that this variable is determined in future studies.

Supplementary material

Table 1 - Retrieved literature data of AgNPs studies, concerning physico-chemical characteristics and species response.

Organism	**Size (nm)**		**Coating**	**LE(C)50**	**Endpoint**	**Reference**
	Provider	**TEM/SEM**		**mg/L**		
Carassius auratus	18		ND	0.530	mortality	(Hedayati et al., 2012)
Carassius auratus	18		ND	0.010	mortality	
Carassius auratus	18		ND	0.100	mortality	
Carassius carassius	30–40	81±2	PVP	0.045	olfaction	(Bilberg et al., 2011)
Ceriodaphnia dubia	20-30		citrate	0.067	immobilization	(Griffitt et al., 2008)
Ceriodaphnia dubia	20-30		metal oxide	0.046	mortality	(Gao et al., 2009)
Ceriodaphnia dubia		25.4	uncapped	4.820 E-04	mortality	(McLaughlin and Bonzongo, 2012b)
Chara vulgaris		10 - 15	ND	0.539	total	(Dash et al., 2012)

					chlorophyll	
Chlamydomonas reinhardtii	25±13		carbonate	0.356	Photosynthetic Yield	(Navarro et al., 2008)
Chlamydomonas reinhardtii	25±14		carbonate	0.089	Photosynthetic Yield	
Chlamydomonas reinhardtii	50		uncapped	1.078	photosystem II	(Dewez and Oukarroum, 2012)
Chlorella vulgaris	50		uncapped	10.000	chlorophyll	(Oukarroum et al., 2012)
Danio rerio	20-30		citrate	7.070	mortality	(Griffitt et al., 2008)
Danio rerio	3	5.9	citrate	10.070	mortality	(Bar-Ilan et al., 2009)
Danio rerio	10	15.3	citrate	13.550	mortality	
Danio rerio	50	51.2	citrate	13.690	mortality	
Danio rerio	100	108.9	citrate	14.810	mortality	
Danio rerio	5-20	5-20	starch	25.000	mortality	(Asharani et al., 2008)
Danio rerio	5-20	5-20	BSA	25.000	mortality	
Danio rerio	10	5-20	ND	250.000	mortality	(Choi et al., 2010)
Danio rerio	30–40	81±2	PVP	0.084	mortality	(Bilberg et al., 2012)
Daphnia pulex	20-30		citrate	0.04	immobilization	(Griffitt et al., 2008)
Daphnia magna	5.94		citrate	0.011	mortality	(Allen et al., 2010)
Daphnia magna	29		citrate	0.011	mortality	(Kennedy et al., 2010)
Daphnia magna	10		ND	0.005	mortality	
Daphnia magna	20		ND	0.005	mortality	
Daphnia magna	49		ND	0.005	mortality	
Daphnia magna	50		ND	0.017	mortality	
Daphnia magna	41		PVP	0.097	mortality	
Daphnia magna	36		EDTA	0.015	mortality	
Daphnia magna	5-25	7.32	citrate	0.004	mortality	(Asghari et al., 2012)
Daphnia magna	16.6	6.47	ND	0.002	mortality	

Daphnia magna	20	17.97	uncapped	0.187	mortality	
Daphnia magna		8.4±2.8	PVP	0.054	mortality	(Blinova et al., 2012)
Daphnia magna		12.5±4	protein	0.049	mortality	
Daphnia magna	35		uncapped	0.1	mortality	(Gaiser et al., 2011)
Daphnia magna	10		uncapped	0.009	mortality	(Hoheisel et al., 2012)
Daphnia magna	20		uncapped	0.0136	mortality	
Daphnia magna	30		uncapped	0.018	mortality	
Daphnia magna	50		uncapped	0.030	mortality	
Daphnia magna	<100		uncapped	0.532	mortality	(Jo et al., 2012)
Daphnia magna	<150		uncapped	1.405	mortality	
Daphnia magna	60		uncapped	0.001	mortality	(Kim et al., 2011)
Daphnia magna	300		uncapped	0.001	mortality	(Kim et al., 2011)
Daphnia magna	60-100		uncapped	7.850 E-04	mortality	(Lee et al., 2012)
Daphnia magna	13.3		citrate	7.980 E-03	mortality	
Daphnia magna	36, 52, and 66		citrate	0.003	mortality	(Li et al., 2010)
Hypophthalmicthys molitrix	18		ND	0.340	mortality	(Hedayati et al., 2012)
Hypophthalmicthys molitrix	18		ND	0.010	mortality	
Hypophthalmicthys molitrix	18		ND	0.100	mortality	
Lemma minor		29.2± 10.9	citrate	0.019	growth (dry weight)	(Gubbins et al., 2011)
Lemma minor		29.2± 10.9	citrate	0.038	Frond Number	
Lemma minor		93.52± 48.6	citrate	0.019	growth (dry weight)	
Lemma minor		93.52± 48.6	citrate	0.042	Frond Number	
Ochromonas danica	1–10		carboxy-functionalized	19.998	growth	(Miao et al., 2010)

Oryzias latipes	28.3		PVP	1.030	mortality	(Wu et al., 2010)
Oryzias latipes	60		uncapped	0.028	mortality	(Kim et al., 2011)
Oryzias latipes	300		uncapped	0.067	mortality	
Oryzias latipes		29.9	PVP	1.380	mortality	(Zhao and Wang 2013)
Oryzias latipes		29.9	PVP	1.120	mortality	
Oryzias latipes		29.9	PVP	0.870	mortality	
Oryzias latipes		29.9	PVP	0.100	mortality	
Perca fluviatilis	30–40	81±2	PVP	0.045	olfaction	(Bilberg et al., 2011)
Pimephales promelas	31-50		ND	9.400	mortality	(Laban et al., 2009)
Pimephales promelas	21-280		uncapped	10.600	mortality	(Laban et al., 2009)
Pimephales promelas	31-50		ND	1.250	mortality	
Pimephales promelas	21-280		uncapped	1.360	mortality	
Pimephales promelas	10		uncapped	0.089	mortality	(Hoheisel et al., 2012)
Pimephales promelas	10		uncapped	0.051	biomass	
Pithophora oedogonia		10 - 15	ND	0.539	total chlorophyll	(Dash et al., 2012)
Pseudokirchneriella subcapitata	20-30		citrate	0.190	growth	(Griffitt et al., 2008)
Pseudokirchneriella subcapitata		25.4	uncapped	0.005	chlorophyll a	(McLaughlin and Bonzongo, 2012a)
Salmo salar			uncapped	0.100	mortality	(Farmen et al., 2012)
Salmo salar			citrate	0.020	gills	
Thamnocephalus platyurus		8.4±2.8	PVP	0.068	mortality	
Thamnocephalus platyurus		12.5±4	protein	0.256	mortality	

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Chapter V: General discussion and final remarks

In this work we showed that AgNPs can have a wide variety of effects at different levels of biological organization. At the sub-individual level, AgNPs affected the proteome expression and the activity of several enzymes in microalgae.

AgNPs, in particular the smaller ones (10 nm) cause phenotypic changes as decrease in the specific growth rate, oxidative stress to the organism, and pose modifications on the proteome expression. In this particular study we hypothesized that toxicity of AgNPs may be driven mostly by dissolution than by size-related effects.

At the individual level, AgNPs delayed the microalgae growth and also reduced the survival, growth and reproduction of the crustacean *D. magna*. Using a simplified food chain, feeding *D. magna* with 300 µg AgNPs/L of pre-exposed algae to silver nanoparticles, revealed a decrease in the *D. magna* survivorship along the exposure, also a significant reduction on offspring production and a significant decrease in the final body weight. In addition, it is suggested that the aqueous exposure might cause more pronounced effects than the dietary exposure. In nature, both types of exposure occur simultaneously, since organisms may face not only contaminated waters but also "contaminated" food.

Despite the significant effects at the sub-individual and individual levels, this study suggests that the predicted environmental concentrations of AgNPs represent no risk to aquatic communities of algae, plants, crustaceans and fish. There are, however, some aspects to have in consideration concerning the effects at the community level. aquatic organisms are not only exposed to AgNPs but also to the silver ion (Ag^{+}), which is recognized as the most toxic silver form to aquatic organisms. Moreover, organisms are exposed to AgNPs both through the water and through the diet, which emphasizes the role of bioaccumulation. In addition, AgNPs accumulate in the sediment, and might be released to the water column under certain environmental conditions, e.g. increased water flow causing the suspension of sediments. Additionally we have stated a non-regular parameter beyond the concentration for the derivation of risk – the surface-area-to-volume-ratio (nm^2 /L) as an alternative metric for evaluation of risk posed by nanomaterials.

This work suggests the use of biochemical markers and protein profiling on microalgae for environmental risk assessment purposes and, thus, will be useful for further investigations,

namely for environmental risk assessment. In the same way, future work should be addressed on the analyses of patterns in biomarkers and protein expression for other contaminants and their response to abiotic factors. Finally, some of the findings of this work might apply also to other metallic nanoparticles, namely the effects at the sub-individual level, the role of dietary exposure and the use of the surface-area-to-volume-ratio as an alternative metric for evaluation of risk posed by nanomaterials.