Universidade de Aveiro Departamento de Biologia
Ano 2016

Cátia Soraia Moreira Monteiro

Efeitos crónicos de nano-ouro em produtores e consumidores primários dulçaquícolas

Long-term effects of nano-gold to freshwater producers and primary consumers

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Long-term effects of nano-gold to freshwater producers and primary consumers

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Isabel Maria Cunha Antunes Lopes, Investigadora Principal do Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho ao meu avô Adriano e ao meu tio Zé.

o júri

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palavras-chave Nanomateriais; Ouro; Efeitos geracionais; Microalgas; *Daphnia magna*

resumo

Os nanomateriais (NMs) tanto podem surgir no ambiente através de fontes naturais (ex. poeira vulcânica, processos de queima), como também ocorrem devido a atividades antropogénicas. A nanotecnologia, que estuda a manipulação de NMs, tem a capacidade de produzir muitos produtos distintos explorando várias características particulares destes materiais, tais como o tamanho e reatividade. Esta capacidade de criar materiais com características específicas tem atraído a indústria de diversas áreas, associadas por exemplo à medicina e eletrónica. Apesar dos benefícios claros dos NMs em várias áreas da sociedade, o crescimento exponencial do seu uso e a sua consequente libertação para o ambiente tem alertado a comunidade científica para os possíveis efeitos adversos que os NMs podem provocar no biota.

O presente estudo pretendeu avaliar os efeitos subletais de nanopartículas de ouro (Au-NP) em três organismos dulçaquícolas, representativos de níveis inferiores de cadeias tróficas. Para atingir este objetivo, foram delineados dois objetivos específicos: i) estudar os efeitos subletais de Au-NP em *Raphidocelis subcapitata* e *Chlorella vulgaris* (produtores) e em *Daphnia magna* (consumidor primário); ii) avaliar os efeitos geracionais das Au-NP nas duas espécies de microalgas. As microalgas foram expostas durante 72h e o consumidor primário durante 21d, a uma ampla gama de concentrações de Au-NP. Foram avaliados efeitos no crescimento populacional e somático e na reprodução, para as microalgas (o primeiro parâmetro) e para *D. magna* (os três parâmetros). Em todas as espécies foram encontradas diminuições significativas ao nível do crescimento populacional comparativamente ao respetivo controlo. No entanto, as Au-NP não induziram qualquer efeito ao nível da reprodução de *D. magna*.

A exposição geracional de *C. vulgaris* a Au-NP provocou uma resposta de aclimatação por parte desta alga, sendo que a partir da terceira geração apresentou um aumento de tolerância à nanopartícula. No entanto, no caso de *R. subcapitata* esta aclimatação não foi observada, e a microalga apresentou maior sensibilidade após ter sido exposta durante quatro gerações a esta nanopartícula.

keywords Nanoparticles; Gold; Long term exposure; Microalgae; *Daphnia magna*

abstract

Nanomaterials (NMs) may occur in the environment through multiple natural sources such as volcanoes (as volcanic dust). However, they may as well occur in the environment originated from anthropogenic activities. Nanotechnology, studies the manipulation of nanomaterials (NMs), producing many different products exploiting some particularities, such as size and reactivity. This capacity to manipulate materials at the nanoscale attracted industry from different areas, namely associated with medicine and electronics. However, the exponential growth in the use of these materials and subsequent release to the environment has alerted the scientific community to the possible adverse effects that NMs may induce to biota.

The main aim of this study was to evaluate the sublethal effects of gold nanoparticles (Au-NP) in three freshwater species representatives of low trophic levels. To achieve this major aim, two specific objectives were delineated: i) assess the sublethal toxicity of Au-NP to producers and a primary consumer and ii) evaluate the generational effects of Au-NP in microalgae. The microalgae were exposed for 72h and the primary consumer for 21d to a wide range of Au-NP concentrations. Alterations in the population and somatic growth, and reproduction were evaluated to microalgae (only the first endpoint) and *D. magna* (the three endpoints). A significant decrease in the population growth was found for all tested species. However, no significant alterations were found in *D. magna* reproduction.

Generational exposure of *C. vulgaris* to Au-NP caused an acclimation response of this alga that became less sensitive to the nanoparticle after generational exposure. Contrarily, for *R. subcapitata* this acclimation was not observed and the microalga was more sensitive to the nanoparticle after being exposed for four generations to Au-NP.

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Chapter 1
General Introduction

Nanotechnology and nanomaterials

The term “nanotechnology” was first used in 1974 by Norio Taniguchi, who described it as consisting of “processes of separation, consolidation, and deformation of materials by one atom or one molecule” (Taniguchi, 1974). At present, and according to the National Nanotechnology Initiative, nanotechnology is defined as “the understanding and control of matter at dimensions between approximately 1 and 100 nanometers” (Subcommittee on Nanoscale Engineering and Nanotechnology, Committee on Technology, & National Science and Technology Council, 2010).

The European Commission define nanomaterials (NMs) as “natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm” (The European Commission, 2011). However, the definition of NMs has been controversial. Currently, new definitions and revisions have been proposed (Kreyling et al. 2010; Rauscher et al., 2015). In the present work, the definition of the European Commission (2011) was adopted. Nanomaterials may exhibit some characteristics that are not present in the corresponding bulk material, such as high surface:volume ratio and high reactivity, which may contribute to an increase on its redox reaction and consequently to the formation of reactive species of oxygen (ROS) when they enter living organisms (Auffan et al., 2009). In addition, other nanosize-associated characteristics have attracted industry, just by changing their geometric configuration, nanomaterials may exhibit different characteristics allowing its use in different industrial areas (Tiwari et al. 2012). The shape anisotropy of gold nanorods is a typical example; while nanospheres of gold possess single surface plasmon resonance, gold nanorods have two plasmon resonance peaks (originated from the longitudinal and transversal radius) that renders them different optical properties and long-term photostability (Burrows et al., 2016). In figure 1 are summarized the main characteristics of nanomaterials that makes them more attractive to industry than their bulk counterparts. The ability to manipulate these characteristics has raised a great interest from the industries, which increased research and production of NMs at global levels. However, these various properties also influence their toxicity to biota (Shin et al., 2015):

- **Small size and large surface area:volume ratio:** the large surface area (greater than the bulk material) and their small size can make them more reactive (Elsaesser & Howard,

2012; Rosenkranz, 2010). Park et al. (2011) studied the effects of different sizes of Ag-NPs (20, 80, 113 nm) on murine peritoneal macrophage cell line. The smallest NPs (20 nm) were more toxic than the others on cytotoxicity, inflammation, genotoxicity and developmental toxicity. Hua et al. (2014) obtained the same correlation between size and toxicity of nanoparticles when they studied the effects of Cu-NPs (25, 50, 100 nm) on zebrafish embryos. The 25 nm Cu-NPs exhibited a higher percentage of mortality than the 50 and 10 nm. Moreover, the percentage of mortality was more relevant at 120h post-fertilization than at 24h. Contrarily, Nasser et al. (2016) showed that survival of *Daphnia magna* was more affected by long gold nanorods (length 146 nm) than short gold nanorods (length 60 nm), thus, suggesting that though it is common for smaller particles to be more toxic, size is not the only characteristic influencing their toxicity.

- **Shape:** Different forms of nanoparticles can cause different toxicity (Albanese et al., 2012). Nasser et al. (2016) evaluated and compared the toxicity of Au nanorods (diameter 25 nm) and nanospheres (diameter 25 nm), negatively and positively charged. Within each category of surface charge, the lethal toxicity of Au to *D. magna* was higher for nanorods than for nanospheres. Ispas et al. (2009) studied the effects of different shapes (dendritic and spheres) of Ni-NPs in zebrafish embryos. For all tests, the dendritic NPs showed higher toxicity than spheres NPs. Prevailing theories suggest that acicular particles induce enhanced toxicity over isotropic ones through obstacle of phagocyte-mediated clearance mechanisms and through the aggravation of proximal cells via mechanical interactions (Brown et al., 2007).
- **Chemical composition:** Nanoparticle toxicity can be influenced by the chemical toxicity of its individual constituents (Lopes, 2012). For example, gold nanoparticles have been considered to be of low toxicity for organisms whether silver NPs have a higher toxicity (Moreno-Garrido et al., 2015). Knowing the toxicity of the main element of the nanoparticle, may help to understand the toxicity of the corresponding NP, especially if the rate of dissolution of ions from the NP is high. However, many elements that are considered non-toxic can become toxic when combined with coating agents, such as gold, which often becomes toxic when combined with the stabilizing agent hexadecyltrimethylammonium bromide (CTAB) (Schachter, 2013).
- **Solubility:** Solubility is a very important factor for aquatic toxicity (Kahru & Dubourguier, 2010). The potential for NPs components to dissolve can influence their persistence in the environment (Misra et al., 2012). Dissolution can lead to the delivery of highly toxic ions,

such as Zn^{2+}, Cu^{2+}, Cd^{2+} and Ag^{+} to biota (Brunner et al., 2006; Misra et al., 2012). Solubility is dependent on particle's chemicals and surface properties and as well by the medium where they are suspended. Several abiotic factors may influence the solubility of NPs, such as pH, temperature, salinity. Studer et al. (2010) exposed on centrifugation Cu-NPs (diameter 20 nm) in Mili-Q water at different pH values. At pH=7.4 the dissolution of Cu-NPs was <0.1 % and in pH=7 the dissolution was 0.3 %. However, when they acidified the pH for the value 5.5, the dissolution of ions increased to 95 %, thud, becoming more available to be uptake by organisms. On the other hand, insoluble nanoparticles, due to their weak capacity of dissolution, if small enough can pass through different biological barriers and accumulated in organisms tissues/organs (Rana & Kalaichelvan, 2013).

- **Aggregation and agglomeration:** Agglomerates are a cluster of primary particles (particles with a defined geometric shape) and/or aggregates whose total surface does not differ from the sum of surface areas of primary particles (Walter, 2013). Agglomerates are connected by weak physical interactions. Aggregates are developed when primary particles form a common crystalline structure, that usually are formed when particles growth together and are aligned side by side. In aggregates, the total surface area is less than the sum of the surface area of the primary particles (Walter, 2013). Yang et al. (2008) observed that carbon nanotubes are mainly accumulated in liver, spleens and lungs in mice without manifesting any acute toxicity, but induce cytotoxicity when carbon nanotubes accumulated begins to aggregate. Kalbassi et al. (2013) studies the effects of colloidal Ag-NPs and suspended powder Ag-NPs in fish. Suspended Ag-NPs in contact with medium form agglomerates. For all tests performed at different hours and organisms (rainbow trout at different stages of the life-cycle), for concentrations until 100 mg/L, the colloidal Ag-NPs were more toxic than suspended powder Ag-NPs agglomerates. They suggested that the agglomeration caused sedimentation of the Ag-NPs, thus eliminating most of these agglomerates from the water column. Furthermore, the formation of agglomerates reduces considerably the surface area available of NPs to bind or react with biological membranes.

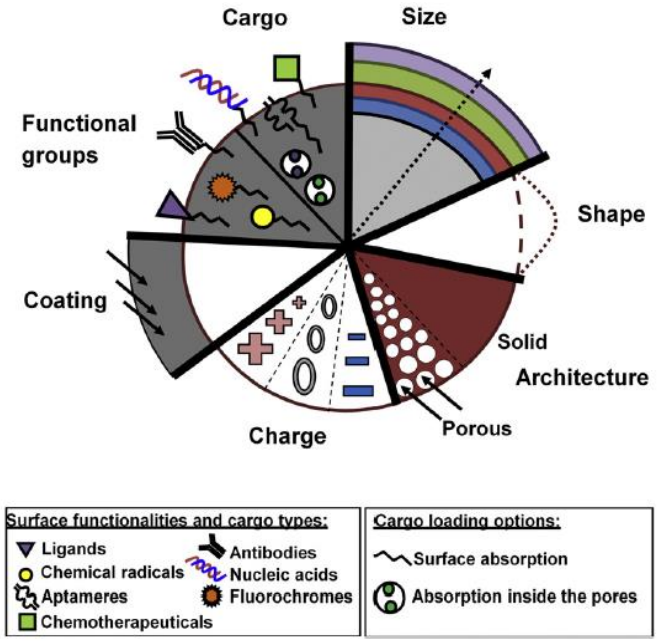

Figure 1: Physical and chemical parameters with particular importance in nanomaterials. (Source: Barkalina et al., 2014)

Nanomaterials in the environment

Fate of nanomaterials in the aquatic systems

Due to the high application and use of engineered nanomaterials in the last decades, it is inevitable that these compounds emerge into the environment via different routes, such as (Klaine et al., 2008; Vale et al., 2016):

- Wastewater treatment plants effluents (WWTP);
- Direct use;
- Deposition from the air compartment;
- Accidental spillages;
- Rainwater runoff.

Methods to determine the presence of NMs in the aquatic environment are still underdeveloped and the expected concentration of many NMs remain mostly unknown (Selck et al. 2016).

In the aquatic environment, the NMs can experiment several t**ransformations** (Figure 2). They can form large aggregates of NMs (**homoaggregation**) whose transport in this environmental compartment is dominated by **sedimentation** (Klaine et al., 2008). The aggregation process can also occur with another particles available in the environment (**heteroaggregation**) such as organic matter (Cupi et al., 2015). Aggregation can modify the toxicity of the NP by decreasing their surface and consequently their reactivity, by increasing their size that reduces their capacity to cross biological barriers (Lowry et al., 2012). Furthermore, aggregation promotes NMs sedimentation, which become more available for benthonic and epibenthonic organisms. However, the particles can suffer **resuspension** due to turbulence of the water returning to the water column and being again available to planktonic and pelagic organisms (Markus et al. 2015). **Dissolution** occur frequently in metal NMs because the formation of soluble metal-oxide, the oxidation of the NMs constituents and the complexation of its constituents with constituents available in the environment easily occurs (Vale et al., 2016). Dissolution reactions can play an important role in the toxicity of nanoparticles. The potential for NPs to dissolve can influence their persistence in the environment and change their biological response. It is already know that dissolution can lead to the delivery of highly toxic ions, such as Zn^{2+}, Cu^{2+}, Cd^{2+} and Ag^{+} to biota (Brunner et al., 2006; Misra et al., 2012). When NM dissolve in the exposure media, speciation of ions with other ligands can be prominent and the uptake path and uptake mechanism of ions in organisms will be different from the NPs themselves. In nanotoxicologic context, an important challenge is important to ascertain whether the toxicity observed is due to the NPs, perhaps as a result of their novel properties, or caused by the release of ions, or a combination of both (Misra et al., 2012).

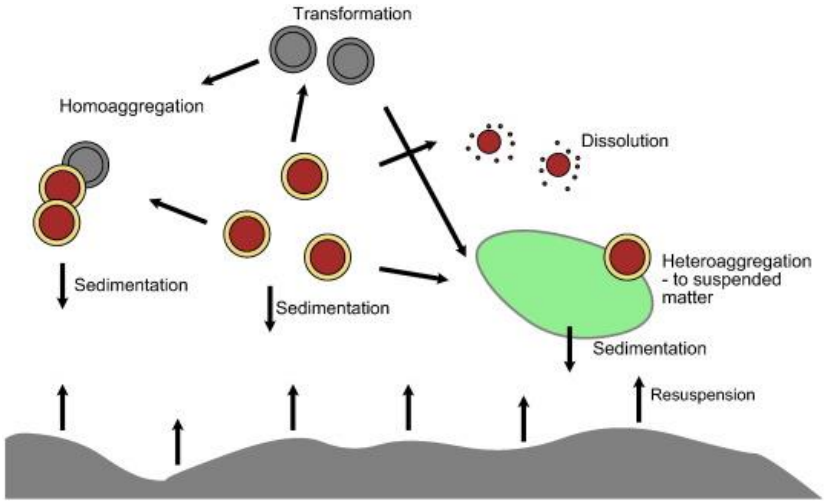

Figure 2: Representation of the main transformation processes of nanomaterials in the aquatic systems. (Source: Markus et al., 2015)

Interactions between nanomaterials and aquatic organisms

After the entrance of NMs in the aquatic environment, through different sources (as previously mentioned), it is expected that they will interact with organisms (e.g. microorganisms, algae, invertebrates, plants, fish) and possibly exert adverse effects on them (Ma & Lin, 2013). Actually, several works have already been published in the last decade illustrating several toxic effects that NMs may induce in biota (Botha et al., 2016; Dědková et al., 2014; Schirmer et al., 2013) Shading, agglomeration and internalization have been identified as playing an important role on NMs toxicity. Shading is particularly harmful for autotrophic organisms, that require light for their survival (Gong et al., 2011; Ma & Lin, 2013; Schwab et al., 2011;).

Algae generally have a cell wall that can play a protective role in supporting the cell structure and defend from the external adverse environments. However, the interaction between NMs and cell wall is poorly understood (Ma & Lin, 2013). But, some authors have reported that some types of NMs can interact directly with algal cells surface through adsorption to the cell wall (Ma & Lin, 2013). Furthermore, the formation of NMs aggregates reduce the amount of light reaching the algal cell and/or blocks the cell wall hindering the acquisition of nutrient and leading to the inhibition of algae growth (Oukarroum et al., 2012; Sadiq et al., 2011; Van Hoecke et al., 2008; Wei et al., 2010).

In animal cells (without cell wall) or in the case of NMs that are capable of crossing the cell wall, NMs enter in contact with the cell membrane. When this occurs, several factors come into play, as some examples (Figure 3):

- NMs with hydrophobic surfaces can be adsorbed in the hydrophobic surfaces zones of the cell membrane (Xiao & Wiesner, 2012);
- Electrostatic attraction may occur when NMs and the cell membrane exhibit different surface charges (Cho et al., 2009);
- Occurrence of hydrogen bond and receptor-ligand interaction (Liang et al., 2016; Yue et al., 2015);
- NMs may disrupt the cell membrane and directly enter the cells (Chen et al., 2009);
- In metallic NMs when the dissociation of ions occurs, they may be transported into the cells via membrane transport channels (Dobson, 2008).
- Endocytosis may occur directly across the membrane (Ma & Lin, 2013) This is the most common route for NMs internalization into cells.

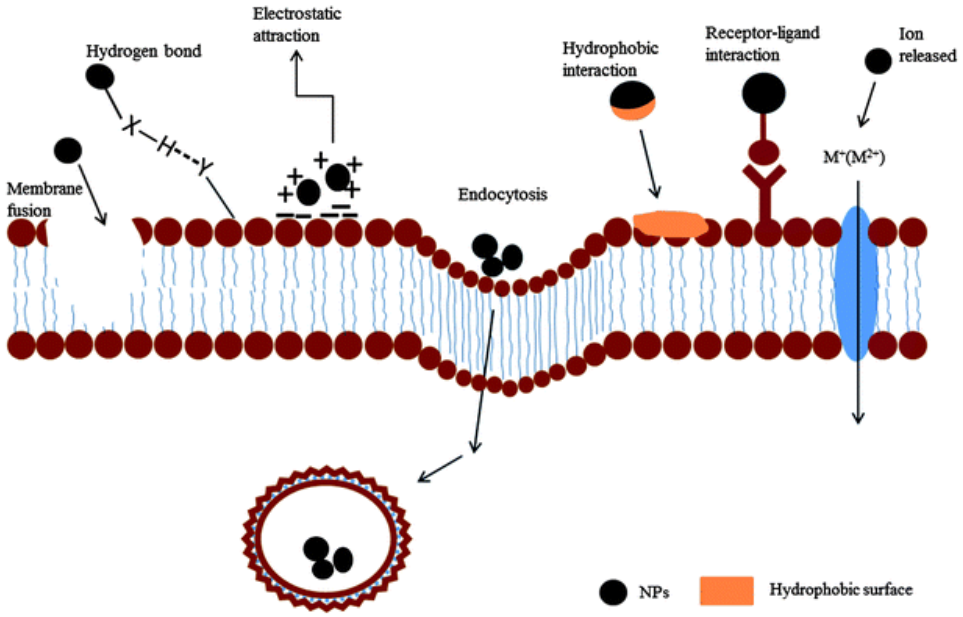

Figure 3: Interactions between nanomaterials and cell membrane (Source: Ma & Lin, 2013)**.**

Gold nanoparticles

Gold nanoparticles (Au-NPs) are a colloidal suspension of gold particles with nanometer size (Tiwari et al., 2011). These nanoparticles have been analyzed and studied due to their unique characteristics – gold nanorods have particular interest due to their anisotropic shape, which makes them attractive in biological imaging and sensing (Zhang et al., 2012). The optical and electric properties of Au-NPs are tunable by changing the size, shape, surface chemistry, or aggregation state. Gold NPs, as any other NPs, can be classified according to their dimensionality, morphology, composition, uniformity and agglomeration/aggregation (Buzea et al., 2007). Figure 4 illustrates the wide diversity of existing types of Au-NPs.

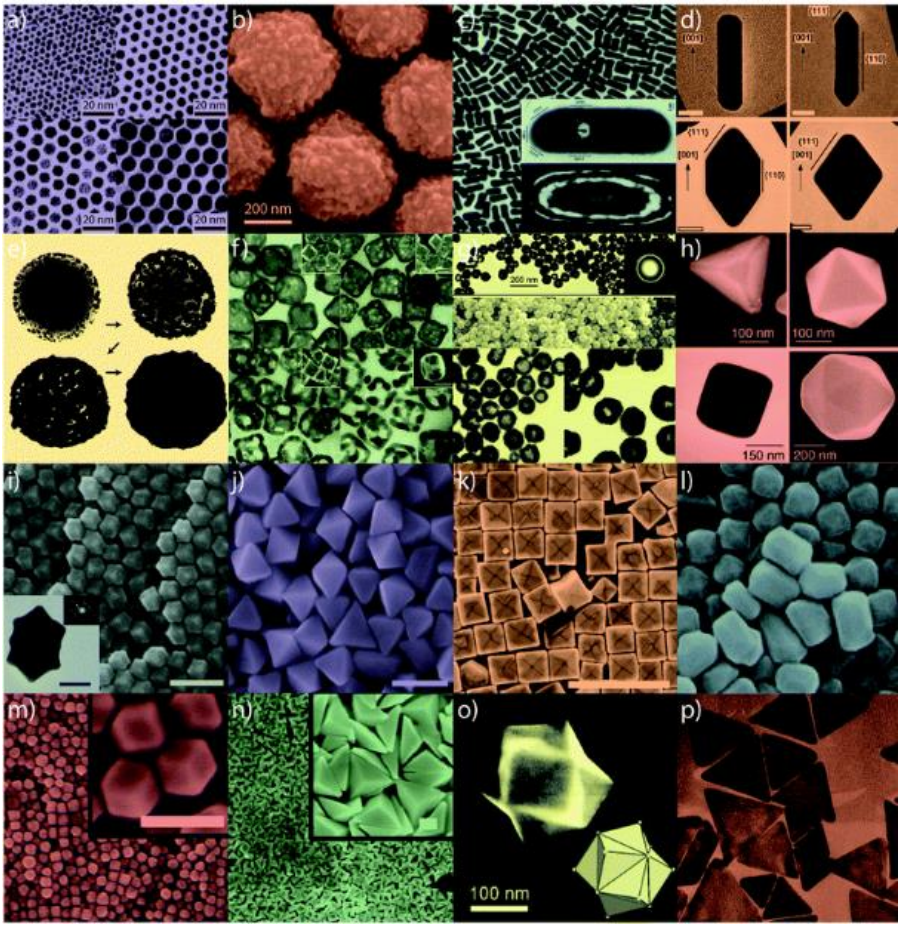

Figure 4: Gold nanoparticles of various sizes and shapes (a) Smal nanospheres, (b) large nanospheres, (c) nanorods, (d) sharpened nanorods, (e) nanoshells, (f) nanocages/frames, (g) hollow nanospheres, (h) tetrahedra/octahedra/cubes/icosahedra, (i) rhombic dodecahedra, (j) octahedra, (k) concave nanocubes, (l) tetrahexahedra, (m) rhombic dodecahedra, (n) obtuse triangular bipyramids, (o) trisoctahedra, and (p) nanoprisms. (Source: Dreaden et al., 2012)

Gold NPs are used in several areas such as (Sharma & Singh, 2016):

- **Electronics** – In the manufacture of connect resistors, conductors, chips (Gutiérrez-Sánchez et al., 2012).
- **Cancer treatment** – Gold-NPs bind to tumor cells and may kill them in a hyperthermia therapy (Jain et al., 2012).
- **Therapeutic agent delivery** – The large surface area to volume ratio allows many molecules to be coupled at Au-NPs surface (Arvizo et al., 2011).
- **Sensors –** As a constituent of various sensors (Yue et al., 2016).
- **Biological imaging applications –** Due to their special optical characteristics (Tong et al., 2009) .

- **Disease diagnostics –** They can behave as biomarkers in the diagnosis of many diseases (Mieszawska et al., 2013).
- **Catalysis –** They are used as a catalyst in number of a chemical reaction, which make them attractive to the industry (Thompson, 2007).

The assessment of Au-NPs toxicity may be quite complex due to its great variety in form, stabilizing coating agents (such as cetyltrimethylammonium bromide-CTAB), physicochemical parameters, incubation conditions, type of specimens used, types of assay, among other factors (Soenen et al., 2011). At the cellular level, spherical Au-NPs may interact with cell membranes, mediated by their strong electrostatic attraction to the negatively charged bilayer (Goodman et al., 2004). Kang et al. (2009) evaluated the effects of spherical Au-NPs (diameter 4, 15, 100, 200 nm) on a mouse cell line at concentrations as high as 200 µg/mL of Au-NPs. The Au-NPs with 4 nm of diameter induced cellular toxicity (assessed by cell counting) at concentrations above 25 µg/mL. Furthermore, the Au-NPs with diameter of 100 and 200 nm induced DNA damage also at concentrations above 25 µg/mL. Dreaden et al. (2012) revised the toxicity of Au-NP and reported that in general these NPs can accumulate in tumor cells and penetrate the cell much faster than other small molecules. Due to their comparable size relative to proteins, Au-NP can selectively perturb and modify cellular processes in ways that small molecules and proteins cannot, allowing them to act as intrinsic drug agents.

At the individual level, Au-NPs may accumulate in the different tissues. Larguinho et al. (2014) reported that the algae *Dunaliella salina* was able to accumulate the major part of spherical Au-NPs to which was exposed (76%) for 24h at concentrations of 0.1–1 nM. García-Negrete et al. (2013) exposed the bivalve *Ruditapes philippinarum* to spherical Au-NPs for different periods of time (3h, 6h, 12h, 24h, 7 days, 14 days, 28days) at concentrations of 6 and 30 µg/L. These authors observed that the accumulation of spherical Au-NP in *R. philippinarum* occurred mainly in the gills and in the digestive gland, for the two tested concentrations. This accumulation was visible even for the lowest exposure period of time (3h). Botha et al. (2016) observed that after a period of 14 days of exposure to concentrations of spherical Au-NPs above 20mg/L, the cladocera *D. magna*, accumulated large amounts of these NPs mainly in its exoskeleton, which may constitute a way to excrete the NP, since these organisms release molts as they grow. Lee et al. (2015) studied the effects in the somatic growth of *D. magna* exposed to colloidal gold nanospheres (size between 8.5 and 12 nm) during 48h and they found an inhibition of growth at concentration of 5 µg/L. Dědková et al., (2014) analyzed the effects on growth of

green algae *Desmodesmus subspicatus* and *R. subcapitata* exposing during 72h to gold nanospheres. These researchers observed significant effects in the growth of both algae and reported EC_{50} of 28 µg/mL and 14 µg/mL for *D. subspicatus* and *R. subcapitata*, respectively.

Model organisms

In this work, three different species were studied, the microalgae (producers) *Raphidocelis subcapitata* and *Chlorella vulgaris* and the microcrustacean (primary consumer) *Daphnia magna*.

Raphidocelis subcapitata

Raphidocelis subcapitata (also known as *Pseudokirchneriella subcapitata*) is a planktonic microalga frequently found in freshwater ponds, lakes and rivers. Its taxonomic classification is (Nygaard et al., 1987):

Kingdom: Plantae
Phylum: Chlorophyta
Class: Chlorophyceae
Order: Sphaeropleales
Family: Selenastraceae
Genus: *Raphidocelis*
Specie: *Raphidocelis subcapitata*

Cells of *R. subcapitata* are semicircular curved and most of time the cells are solitary (Figure 5; Aruoja, 2011). The reproduction of this microalgae is by division of the cell into 2, 4 or 8 autospores (Nygaard et al. 1986).

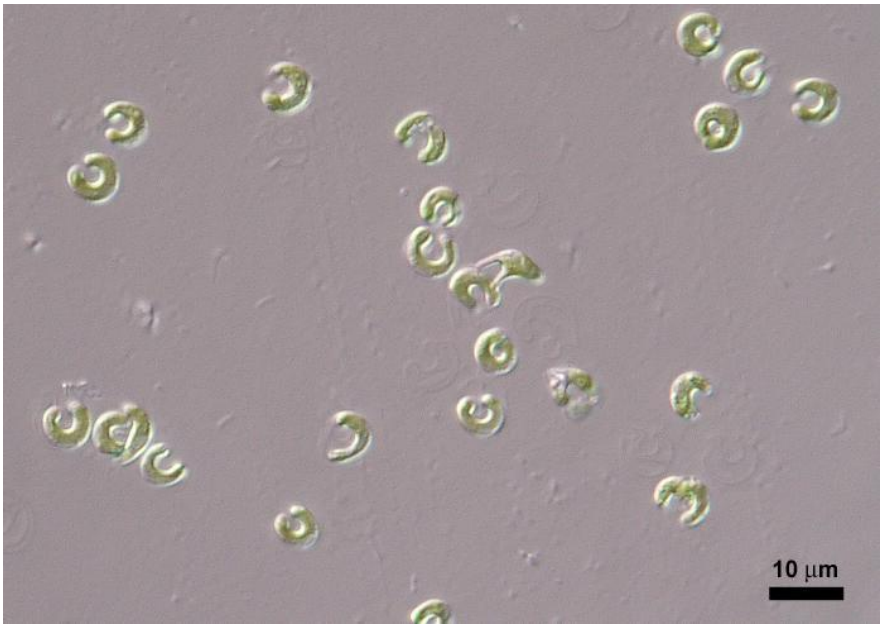

Figure 5: *Raphidocelis subcapitata* cells view in the optical microscope. (Source: Culture Collection of Autotrophic Organisms, 2013b)

This species has been widely used in nanotoxicology as a model species. As an example, Nogueira et al. (2015) studied the effects of four metal nanoparticles – TiO_2, Fe_2O_3, NiO (10-20 nm) and NiO (100 nm) to a battery of aquatic organisms. For *R. subcapitata*, no significant effects on growth occur after being exposed to 20.0 mg/L of nano-Fe_2O_3. The two types of NiO nanoparticles induced a significant decrease in the growth rate of *R. subcapitata*, with an EC_{50} of 8.24 mg/L and of 15.2 mg/L for NiO (100 nm) and NiO (10-20 nm), respectively. Exposure to nano-TiO_2 caused an increase in growth rate comparatively to control at concentrations of 16 and 20 mg/L. Regarding the effects of nano-TiO_2 in microalgae are still controversial.

Studies with another nanoparticles has been performed and showed as well the sensitivity of these microalgae to nanoparticles (Aruoja, 2011; Radix et al., 2000).

Chlorella vulgaris

Chlorella vulgaris, as *R. subcapitata*, is a planktonic microalga frequently found in freshwater ponds, lakes and rivers. Its taxonomic classification is (Beyerinck, 1900):

Kingdom: Plantae

Phylum: Chlorophyta

Class: Trebouxiophyceae

Order: Chlorococcales

Family: Chlorellaceae

Genus: *Chlorella*

Specie: *Chlorella vulgaris*

Cells of *C. vulgaris* are globular with a diameter between 4-10 nm (Figure 6; Geiger, 2014).

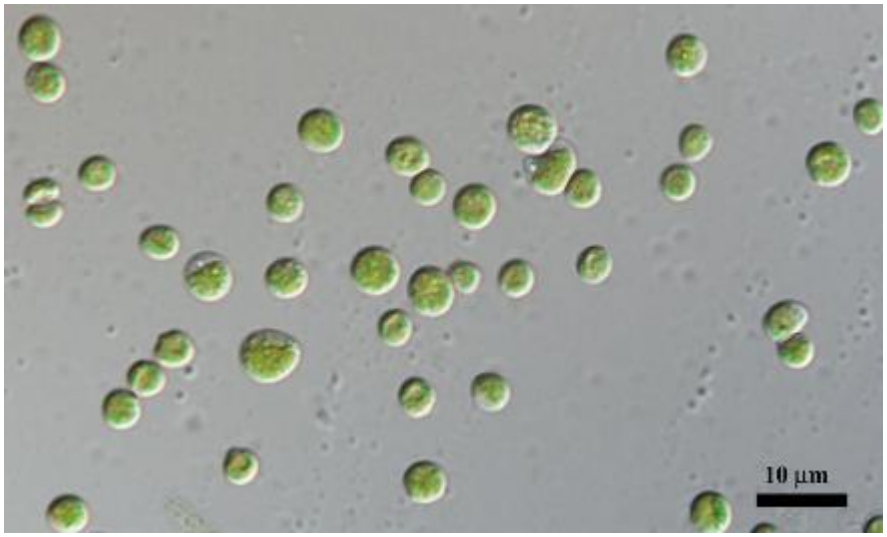

Figure 6: *Chlorella vulgaris* cells view in the optical microscope. (Source: Culture Collection of Autotrophic Organisms, 2013a)

Chlorella vulgaris, as *R. subcapitata*, have been used in many studies to assess the toxicity of NPs. Oukarroum et al. (2012) studied the effects of 50 nm silver NPs in *C. vulgaris*. Silver NPs at a concentration between 0-10 mg/L exerted a negative effect on *C. vulgaris* manifested by a strong decrease on chlorophyll values, viable algal cells and increase of reactive oxygen species formation. Gong et al. (2011) reported that nickel oxide NPs caused a significant effect on *C. vulgaris* growth, with an EC_{50} of 32.28 mg/L. Moreover, under stress of nickel oxide NPs, until 50 mg/L of concentration, cells of *C. vulgaris* showed plasmolysis, cytomembrane breakage and thylakoids disorder.

Daphnia magna

Daphnia magna is a well-studied planktonic cladoceran that belongs to the phylum Arthropoda. *Daphnia magna* possesses an exoskeleton, jointed limbs and a hemocoel as primary internal cavity (Figure 7) (Rosenkranz, 2010). The taxonomy of *D. magna* is (Boxshall, 2015):

Kingdom: Animalia

Phylum: Arthropoda

Class: Branchiopoda

Order: Diplostraca

Family: Daphniidae

Genus: *Daphnia*

Species: *Daphnia magna*

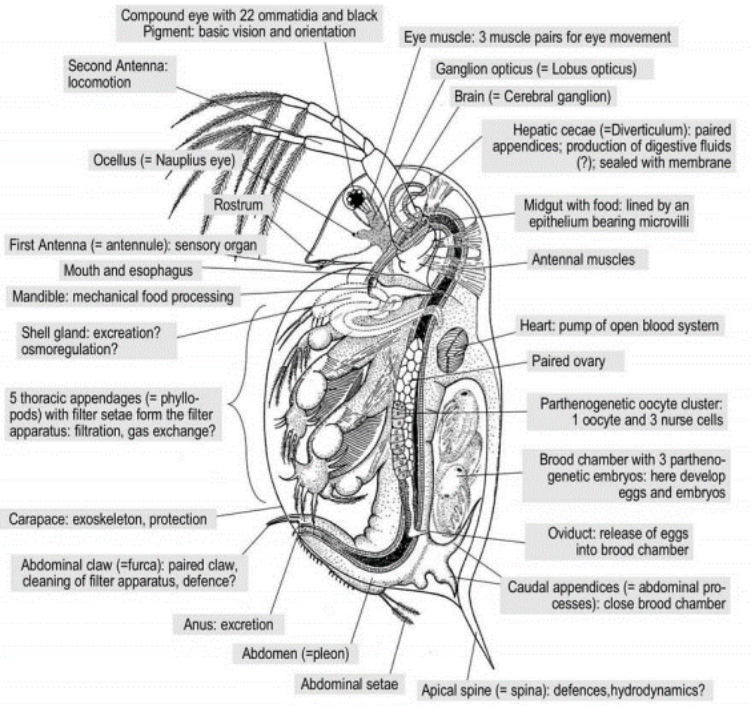

Figure 7: Schematization of *Daphnia* morphology. This figure shows an adult female with parthenogenetic embryos in her brood chamber. (source: Ebert, 2005)

Daphnia magna reproduces through cyclic parthenogenesis (Rosenkranz, 2010). During the asexual phase, *D. magna* reproduces by parthenogenesis, which allows to keep the same clone in laboratory for several generations (Tavares, 2014). The asexual life-cycle occurs in four

distinct stages: the egg formation, juvenile (3-5 juvenile phases between molting), adolescent (more one phase between molting) and adult (6-22 phases between molting) (Mitchell, 2001; Rosenkranz, 2010). Under unfavorable environmental conditions (e.g. low temperatures, low food levels) females may switch to sexual reproduction, by producing males and haploid eggs. The graphic representation of *D. magna* life cycle can be found in Figure 8.

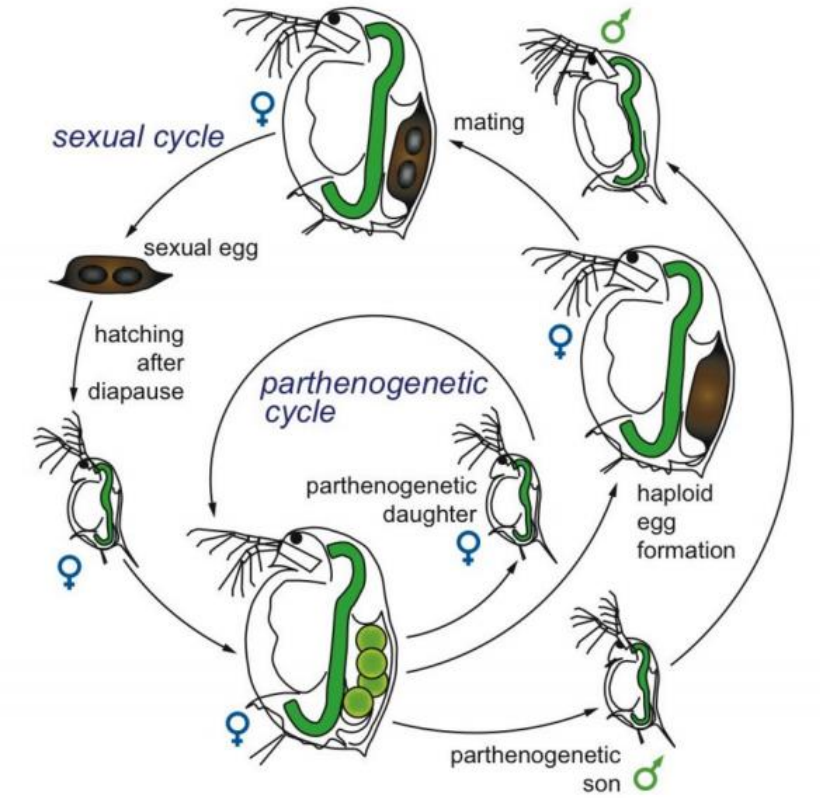

Figure 8: Representation of *Daphnia magna* life cycle
(source: Ebert, 2005)

Daphnia magna has been used in ecotoxicology since 1960s (Botha et al., 2016), being the most widely used model species in ecotoxicity studies. Being a primary consumer, it holds an intermediate position in the transfer of energy and biomass from the producers to other consumers (Baun et al., 2008). Another particularity of *D. magna* is to be a filter feeder organism, allowing them to filter large volumes of water compared to body size (Baun et al., 2008). This capacity has been explored in some studies that found nanoparticles in the digestive tract of *D. magna* (Baun et al., 2008; Matos et al., 2009).

Lovern & Klaper, (2006*)* assessed the lethal effects of filtered nano TiO_2 and C_{60} in *D. magna* and observed that the nano-C_{60} ($LC_{50,48h}$=0.46 mg/L) exerted a higher toxicity

comparatively to nano-TiO_2 ($LC_{50,48h)}$=5.5 mg/L). Latter, these same authors assessed the lethal effects of fullerene (C_{60}) and nano-TiO_2 on neonates of *D. magna* after an exposure for 48h to these NPs (Lovern & Klaper, 2008). For daphnids exposed to C_{60}, mortalities of 12% and 100% was registered at 40 µg/L and 880 µg/L of C_{60}, respectively. Regarding nano-TiO_2, the lowest concentration tested (200 µg/L) caused mortality of daphnids below 2 %. However, 100% of mortality occurred when *D. magna* neonates were exposed to 10 000 µg/L of nano-TiO_2. Oberdörster et al. (2006) studied the effects of long-term exposure to 2.5 mg/L of C_{60} in *D. magna*. These authors observed a significant decrease in the total number of offspring produced by daphnids and a delay in time to molting. Botha et al. (2016) studied the same parameters but by exposing *D. magna* to spherical Au-NP and they did not found significant effects in offspring production and molting patterns at concentrations equal or below 20 mg/L.

Aims

The general aim of the present work was to evaluate the sublethal effects of gold-nanorods to freshwater organisms. To attain this major goal, two specific objectives were delineated: (i) to assess the sublethal toxicity of gold-nanorods to producers and a primary consumer and ii) to evaluate the generational effects of gold-nanorods in microalgae.

This work was divided into four chapters. In the first chapter a brief contextualization of the thematic of the present work is provided. The state of the art is presented followed by the objectives. The second chapter corresponds to the work developed to attain the first specific objective. The sublethal effects caused by exposure to gold nanorods was assessed in three freshwater species, representative of different trophic levels: *Chlorella vulgaris* and *Raphidocelis subcapitata* (producers) and *Daphnia magna* (consumer). The third chapter describes the work developed to attain the second specific objective. Taking into account the results obtained in the second chapter, the multigenerational effects of gold nanorods in *Chlorella vulgaris* and *Raphidocelis subcapitata* were evaluated to try to predict the impact that long-term exposure to these chemicals may have on the trophic chain. Finally, in the fourth chapter, the major finding and conclusions of this work are summarized and research lines for prospective work are suggested.

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Chapter 2
Sublethal effects of gold nanorods to algae and cladocera

Abstract

Gold nanorods (Au-NP) have been deeply studied aiming its application, among others, in diagnostic procedures. The aim of this study is to evaluate the sublethal effects caused by exposure to Au-NP on species representative of two trophic levels: *Chlorella vulgaris* and *Raphidocelis subcapitata* (as primary producers), and *Daphnia magna* (as primary consumer). The three species were exposed for 72h and 21 days to serial dilutions of Au-NP, respectively for the microalgae and *D. magna*. Effects on time to release the first reproduction, total reproduction and somatic growth of *D. magna* and in the population growth rate of all tested species were monitored. For the two species of microalgae the concentrations of Au-NP inducing 20 and 10% of effect in the population growth rate were as follows: $EC_{20,72h}$= 39 µg/L and $EC_{20,72h}$= 79 µg/L for *R. subcapitata* and *C. vulgaris*, respectively; and $EC_{10,72h=}$ 22 and $EC_{10,72h}$= 21 for *R. subcapitata* and *C. vulgaris*, respectively. As for the effects on the somatic growth of *D. magna*, a statistically significant decrease for all tested Au-NP concentrations was observed, being computed an $EC_{10,21d}$ of 2.45 µg/L. However, no significant effects were observed in the total number of released neonates between control and Au-NP exposed groups. The low levels at which the Au-NP exerted sublethal effects in the studied species suggest that its release in a long run into freshwater ecosystems may constitute an ecological risk.

Keywords

Chlorella vulgaris, *Raphidocelis subcapitata*, *Daphnia magna*, gold nanorods, CTAB

Introduction

Nanotechnology explores particular features of nanosize, like increased reactivity, that make NMs attractive to produce a vast panoply of consumer products (Völker et al., 2013). Specifically, gold nanoparticles (Au-NP) have received a special interest from several industrial areas due to their singular characteristics such as extraordinary optical and electronic properties, high stability, biological compatibility, controllable morphology, size dispersion and facility of its surface functionalization (Conde, 2013). Anisotropic nanoparticles – non-spherical structures (such as nanorods and nanostars) can be used in a number of important applications ranging from catalysis to sensing to optics. These nanoparticles have plasmon resonances (local of collective oscillation of electrons) that can be tuned by their size and morphology (Chandra et al., 2016; Novikov et al., 2014). Gold nanorods have a transversal and a longitudinal plasmon – one along the short axis (transversal) and the other along the long axis (longitudinal) (Fratoddi et al., 2015). These characteristics are widely used in medicine mainly to develop diagnostic procedures. Actually, Au-NP based diagnostic devices are already being commercialized or are at evaluation stages of clinical trials and Au-NP based therapeutics and theranostics (combined diagnostic and treatment modality) are in the phase of research and development. Though these advantages are associated with diverse benefits to the society, they may as well be associated with unwanted effects in the environment. Therefore, understanding the adverse effects that Au-NP may pose in the environment is mandatory since, due to its diverse application in products to be used/consumed by society it is predicted that it will be release in the environment (Dědková et al., 2014).

The toxicity of Au-NP may be influenced by several factors, namely their intrinsic nature and capacity to form larger aggregations (in size and shape), the route of exposure, exposure time, the interactions with mechanisms involved in the physiological process of uptake, among others (Lapresta-Fernández et al., 2012; Dědková et al., 2014). Some studies have already been performed to assess the toxicity of Au-NP to freshwater biota, namely for cladocerans (Botha et al., 2016; Galindo, 2014; Lovern & Klaper, 2006; Völker et al., 2013), green algae (Aruoja, 2011; Galindo, 2014; Geiger, 2014), fishes (Bar-Ilan et al., 2009; Farkas et al., 2010), mollusks (Renault et al., 2008; Tedesco et al., 2010), among others. For example, Dědková et al., (2014) evaluated the effects of gold nanospheres on the growth of two green algae (*Desmodesmus subspicatus* and *R. subcapitata*) after an exposure of 72h. These researchers observed significant effects in the

growth of both algae and reported EC_{50} of 28 000 µg/L and 14 000 µg/L for *D. subspicatus* and *R. subcapitata*, respectively. Nasser et al. (2016) reported that both surface charge and geometric configuration influence the toxicity of Au-NP. These authors evaluated the effects of different Au-NPs (nanospheres with diameter of 25 nm; short nanorods with diameter of 25 nm and length of 60 nm; long nanorods with diameter of 25 nm and length of 146 nm), positively and negatively charged, on *D. magna*. After 24h of exposure to these NP the authors found that, regardless of shape, positively charged Au-NPs were more toxic, with an EC_{50}=6 µg/L and EC_{50}=18 µg/L for short nanorods and nanospheres, respectively. For the negatively charged Au-NPs a maximum of 40% of mortality was only observed at 50 000 µg/L. The authors suggested that such highest toxicity of positively charged NP was due to the highest attraction for the negatively charged phospholipid bilayer of the cell membrane. Regarding the influence of geometric configuration, these authors reported spheres to be the most toxic followed by short rods and long rods. Corroborating the results obtained at the individual level, Nasser et al. (2016) also reported that the negatively charged gold nanospheres induced minimal production of reactive oxygen species (ROS), while positive nanospheres induced a significant production of ROS. Jensen et al. (2016) evaluated the uptake of spherical Au-NP (size 10 nm) into the *D. magna* gut lumen and also the potential internalization into gut cells, by exposing this species to 400 µg/mL for 24h. At the end of exposure, the authors verified Au-NP attached to the intestinal epithelial cells of *D. magna*.

Though some studies on the toxicity of Au-NP to aquatic biota have already been carried out, most of them deal with short-term exposures. Only a few studies have studied chronic or life-cycle effects of these NP to aquatic biota (Baun et al., 2008). For example, Botha et al. (2016) studied the sublethal effect of Au-NP in reproduction the of *D. magna*. After 14 days of exposures, no significant effects were observed at concentration equal or below 20000 µg/L. Bozich et al. (2014) exposed *D. magna* for 21 d to Au-NP coated with different stabilizing agents: trisodium citrate-Cit-Au-NP, poly(allylamine) hydrochloride-PAH-Au-NP, mercaptopropionic acid MPA-Au-NP and trimethylammonium bromide-CTAB-Au-NP. Initial particle charge significantly impacted the observed toxicity, with positively-charged particles (PAH and CTAB–Au-NP) being more toxic than their negatively-charged counterparts. The CTAB-Au-NP affected negatively the reproduction of *D. magna* at 10 µg/L, while PAH–Au-NP significantly affected daphnid reproduction at 5 µg/L. The Cit–Au-NP and MPA-Au-NP affected *Daphnia magna* reproduction at concentration of 25000 µg/L. The possible reason that PAH and CTAB–Au-NPs were more toxic than their negatively-charged Au-NP is potentially due to these positive particles having a high affinity for the cellular membranes. In addition to the need of generating data on the sublethal effects of Au-NP, the

examples given above highlight the difficulty on characterizing the ecological risk of the group of Au-NP since its toxicity is influenced by several properties (e.g. size, shape, charge, capping agents), thus, suggesting the need to perform ecotoxicological evaluations to the diverse range of Au-NP being produced. Aiming to contribute to fill some of these knowledge gaps, the aim of this study was to evaluate the sublethal effects of gold nanorods to three freshwater species, representative of different trophic levels: *Chlorella vulgaris* and *Raphidocelis subcapitata* (producers) and *Daphnia magna* (consumer).

Materials and methods

Tested substance

Gold nanorods (Au-NP) (A12-10-780) were supplied by Nanopartz™ (Salt Lake City, UT, USA) as dispersion in deionized water with hexadecyltrimethylammonium bromide surfactant capping agent (CTAB) (the concentration of CTAB is maintained below 10mM) with a concentration of Au-NP of 35 µg/ml. The primary size (given by the manufacturer) of Au-NP in the dispersion was as follows: diameter=10 nm, length=38 nm. The surfactant hexadecyltrimethylammonium bromide (CTAB), used as a capping agent of the Au-NP, was supplied by Sigma-Aldrich® as a powder (purity of 95%).

Test species

Cultures of *Raphidocelis subcapitata* and *Chlorella vulgaris* were maintained at 20˚C with continuous light (fluorescent light tubes: OsramL36W/10) and aeration. Four-day inoculates, i.e. in the log growth phase of the algae cultures, were used to run the ecotoxicological assays. Woods Hole MBL culture medium was used and prepared according to Stein, 1973. The medium, such as all the material used to prepare the cultures and assays, were previously sterilized in an autoclaved at 121°C and 1 Bar, for 30 minutes. *Raphidocelis subcapitata* and *C. vulgaris* are two species of freshwater green microalgae representatives of primary producers.

Daphnia magna BEAK culture was maintained in ASTM medium (ASTM, 2002) and held in 1L glass vessels with 16:8h light:dark cycle, under controlled temperature (19-22˚C). Medium was changed three times per week and organisms were fed daily with the green algae *Raphidocelis*

subcapitata (density=3.0×10^5 cell/mL/daphnia) and organic additive Marinure 25 (an extract from the algae *Ascophyllum nodosum*; Pann Britannica Industries Ltd., Waltham Abbey, UK) (Baird et al., 1989). All cultures were maintained under asexual reproduction (parthenogenesis), which allowed maintaining in the laboratory the same clone for several generations. Cultures were renewed with neonates from third, fourth or fifth brood.

Both the species of microalgae and *D. magna* have been recommended as standard species for testing of chemicals. They are easily maintained in the laboratory, very sensitive to different contaminants and they have short life cycles (Martins, 2010; Tavares, 2014).

Gold nanorods characterization

The physical characterization of the stock Au-NP suspension was performed. The surface charge, the zeta potential and the hydrodynamic diameter of the Au-NP in dispersions were determined in a Zetasizer Nano ZS with Zetasizer Software (Malvern ZetaSizer, 2013). Ultraviolet/visible/infrared (UV/Vis/IR) Jasco U-560 UV–Vis spectrophotometer was used to determine the spectra of light that is absorbed and scattered by the Au-NP dispersions (NanoComposix, 2012).

72h-growth inhibition assays with microalgae

The effects of Au-NP on the growth rate of microalgae was assessed by exposing *R. subcapitata* and *C. vulgaris* to this NP according to the OECD Guideline 201 (OECD, 2004). Preliminary assays were carried out to select the concentration range to which algae were exposed. *Raphidocelis subcapitata* was exposed to seven concentrations ranging from 8 µg/L to 53 µg/L, using a dilution factor of 1.3, plus a control that consisted in medium MBL. For *Chlorella vulgaris* eight concentrations of Au-NP were tested ranging from 11 to 90, using a dilution factor of 1.3, plus a control of MBL. To evaluate the effects of the Au-NP stabilizer CTAB, both algae were also exposed to the concentration of CTAB present in the highest tested concentration of Au-NP (90 µg/L for *C. vulgaris* and 53 µg/L for *R. subcapitata*). All assays were conducted at 23±1°C of temperature, under continuous light of 4000 lux, approximately. Assays were performed in 24-well plates and eight replicates were made for each concentration and control. In order to minimize evaporation, the outer wells of the plate were filled with 1 mL of autoclaved distilled water and only the inner wells were filled with test solutions. The test wells were filled with 900µl of: MBL solution solely (control), MBL with Au-NP or with MBL with CTAB. To all test

wells were added 100µl of algae inoculum, 4-5 days old, at a concentration of 10^5 cell/mL, to start the test with a cell density of 10^4 cell/mL (Figure 9).

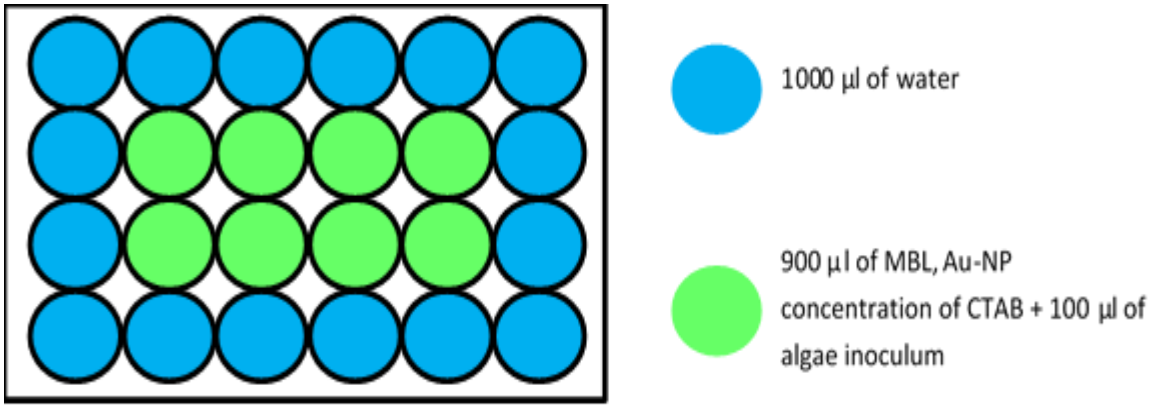

Figure 9: Scheme illustrating how the 24-well microplates were filled with test solutions to run the assays with the microalgae.

To avoid the settling of algae and subsequent shadow effects on their growth, all test plates were re-suspended for a few minutes every day on an orbital shaker. After 72h of exposure the assay ended and absorbance (ABS) was measured for each replicate in a spectrophotometer (Jenway, 6505 UV/Vis) at 440nm, and converted in cell density per volume according to the following equations:

$$Cell/mL = -17107.5 + \text{Abs}440 \times 7925350$$ (*R. subcapitata;* r^2=0.98; p≤0.05)

$$Cell/mL = -155820 + \text{Abs}440 \times 13144324$$ (*C. vulgaris;* r^2=0.91; p≤0.05)

The population growth rate (r) was calculated according to the following equation:

$$r = \frac{\ln N_F - \ln N_I}{t}$$

where N_F is the mean number of algae at the end of assay (cell/mL), N_I is the mean number of algae at the start of the assay (cell/mL) and t is the time of exposure (days).

The percentage of growth inhibition was calculated according to:

$$I(\%) = ((N_C - N_{con})/N_C) \times 100$$

where N_C is the mean number of algae in control and N_{con} is the mean number of algae in respective concentration.

Reproduction assays with *Daphnia magna*

The effects of Au-NP in the reproductive output of *D. magna* was assessed by preforming the 21-day reproduction assay according to the guideline OECD 211 (OECD, 2012). A preliminary assay was carried out to establish the following range of Au-NP concentrations to which *D. magna* were exposed: 1, 1.4, 1.96, 2.74 and 3.84 µg/L. Neonates (<24h old) were exposed to these concentrations and to a control (ASTM medium). Ten neonates were exposed individually in 50-mL glass vessels containing 40 mL of the test solution, per treatment. Organisms were fed daily with *R. subcapitata* (density=3.0×10^5 cell/mL/daphnia) and test medium was changed three times per week. Exposure occurred under controlled conditions of 16:8 light:dark and of temperature between 19˚C-22˚C. After females started releasing the broods, neonates were counted on a daily basis and the time to the first brood was monitored. At the end of 21 days of exposure, the females of each replicates were measured in a binocular microscope (Zeiss mod. Stemi 2000C). Twenty neonates, collected from the same pool from where neonates were sampled to initiate the assay, were measured at the beginning of the assay. Daily somatic growth rate ($mmday^{-1}$) was calculated according to the following formula:

$$k\ (mmd^{-1}) = \frac{\ln(lf) - \ln(li)}{\Delta t}$$

Where k corresponds to growth rate (d^{-1}), li is the initial size (mm), lf is the final size (mm) and Δt is the time interval (days) (Antunes et al. 2003; Galindo, 2014).

The intrinsic rate of population increase (*r*) was computed by using the Euler-Lotka equation,

$$1 = \sum_{x=0}^{n} e^{-rx}\, l_x m_x$$

Where *r* is the intrinsic rate of population increase (d^{-1}), x is the age class (days), l_x is the probability of surviving to age x and m_x is the fecundity at age x. The estimation of standard errors was calculated according to the jack-knifing method (Meyer et al. 1986).

During the assays the following parameters were measured, before and after renewing the test media: conductivity (Wissenschaftlich Technische Werkstätten-WTW conductivity 440i, Weilheim, Germany) pH (WTW pH 330i) and dissolved oxygen (WTW OXI 330i).

Data analysis

For the test with *R. subcapitata* and *C. vulgaris*, the effective concentration inducing 20% and 10% ($EC_{20,72h}$ and $EC_{20,72h}$, respectively) of growth inhibition was calculated with STATISTICA 8.0 software™ (Zar, 1999) by fitting the data to a logistic model.

After confirmed the ANOVA assumptions (Kolmogorov–Smirnov test for normality of data, and Bartlett's test for homoscedasticity of variance), a one-way ANOVA analysis was performed to determine if there were significant differences between treatments regarding growth rate (algae and daphnids), reproduction and somatic growth. The Dunnett's multiple comparison test was performed to compare results between Au-NP and the control (Zar, 1999). To evaluate significant differences in time to release the first brood between treatments an ANOVA on ranks was carried out followed by the Dunn's test.

To assess the effects of CTAB on the microalgae species, a student-t test was performed to compare responses between CTAB treatment with the control. A student-t test was also performed to evaluate the differences between growth of algae exposed to CTAB and to the highest Au-NP tested concentration. All analyses were done using the SigmaPlot 11.0 software™.

Results

Gold nanorods characterization

The measured value of zeta potential of the stock solution, was 71.6 mV, the conductivity 0.176 mS/cm, the hydrodynamic diameter 30.81 nm and the polydispersity index was 1.0. The value for absorbance of stock solution had a principal absorption peak at 780 nm.

72-growth inhibition assay with microalgae

Concentrations of Au-NP above 24 µg/L induced a significant decrease in the growth rate of *C. vulgaris*, comparatively to the control (Dunnett's test: $p<0.001$, Figure 10). Nevertheless, the percentage of growth inhibition relatively to the control never exceeded 25% (

Figure **11**). The concentration of Au-NP inducing 20% and 10% of growth inhibition in this algal species was 79 µg/L (95% confidence limits-CL: 57-100 µg/L) and 21 µg/L (95% CL: 10-32 µg/L), respectively.

The tested concentration of CTAB induced a significant decrease of 15.5% in the growth rate of *C. vulgaris* relatively to the control (Student-t test: p=0.001). No significant differences were observed in the growth rate of this alga when exposed to CTAB compared to the highest tested concentration of Au-NP (CTAB vs 90 µg/L; Student-t test: p=0.442).

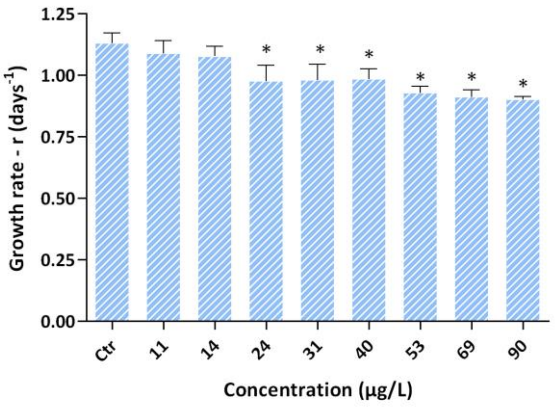

Figure 10: Average of daily growth rate for *Chlorella vulgaris* after being exposed, for 72h, to a range of concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the control (p=<0.001).

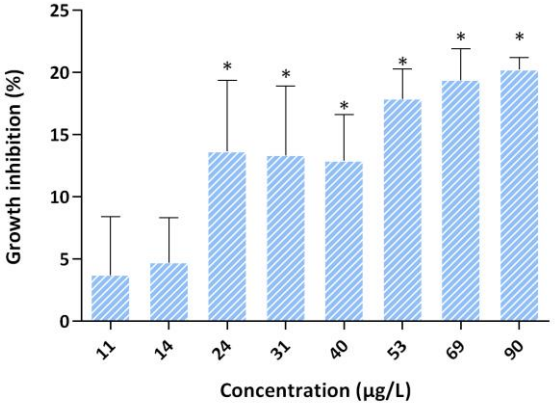

Figure 11: Percentage of growth inhibition relatively to the control for *Chlorella vulgaris* after being exposed, for 72h, to a range of concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the respective control (p=<0.001).

Concentrations of Au-NP above 31 µg/L induced a significant decrease in the growth rate of *R. subcapitata*, comparatively to the control (Dunnett's test: p<0.001; Figure 12). The highest observed percentage of growth inhibition relatively to the control was 29 %, at the highest tested Au-NP concentration (53 µg/L; Figure 13). The concentrations of Au-NP inducing 20% and 10% of growth inhibition in this algal species were 39 µg/L (95% CL: 30-48 µg/L) and 22 µg/L (95% CL: 13-33 µg/L), respectively.

The tested concentration of CTAB induced a significant decrease of 15.5% in the growth rate of *R. subcapitata* relatively to the control (Student-t test: p=0.012). Significant differences were also observed between the growth rate of *R. subcapitata* exposed to CTAB and to the highest tested concentration of Au-NP (CTAB vs 53 µg/L; Student-t test: p=0.039) - the percentage of inhibition being higher for Au-NP.

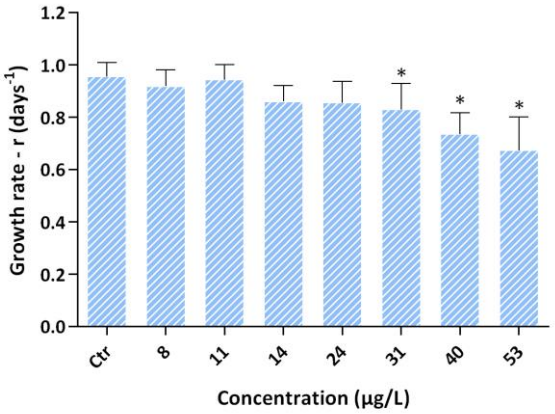

Figure 12: Average of daily growth rate for *Raphidocelis subcapitata* after being exposed, for 72h, to a range of concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the control (p=<0.001).

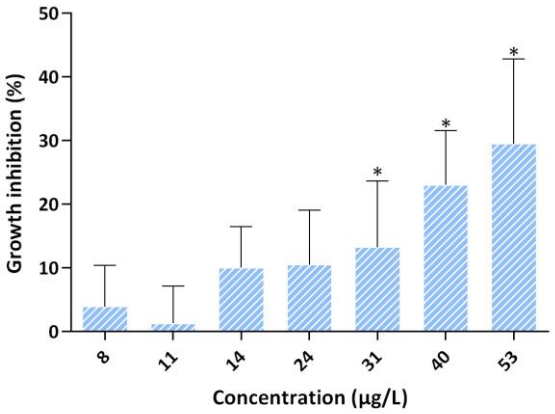

Figure 13: Percentage of growth inhibition relatively to the control for *Raphidocelis subcapitata* after being exposed, for 72h, to a range of concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the respective control (p=<0.001).

21d-reproduction assay with *Daphnia magna*

No relevant changes were observed in the physico-chemical parameters that were monitored along the assay. Dissolved oxygen was always above 20 mg/L. The pH of the medium varied within 7.7 and < 8.5. The temperature was maintained between 21 ˚C and 23 ˚C.

Exposure to Au-NP induced a significant change in time to release the first brood in daphnids exposed to 1.96 µg/L, which released the first brood approximately one day later than the control (Dunn's test: p<0.05; Figure 14). Daphnids exposed to 1.96 and 3.84 µg/L of Au-NP released, in total, less neonates than the control (Dunnett's test: p<0.05; Figure 15). However, for the intrinsic rate of population growth increase significant differences relatively to the control were only detected at 1.4 and 1.96 µg/L of Au-NP (Dunnett's test: p<0.05; Figure 16).

All tested concentrations of Au-NP caused a significant reduction in the somatic growth rate of the daphnids (Dunnett's test: p<0.05; Figure 17), though these reductions never exceeded 13% (only the two highest tested concentrations caused reduction in growth above 10%). The concentration causing 10% ($EC_{10,21d}$) of reduction in the somatic growth rate was 2.45 µg/L (95% CL: 1.43-3.47 µg/L).

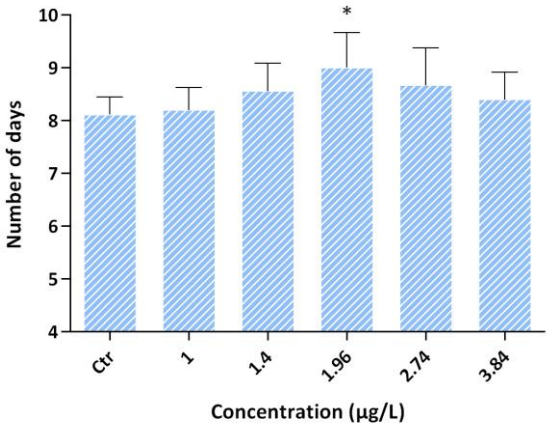

Figure 14: Average of time (in days) until the release of the first brood by females of *Daphnia magna* after being exposed for 21 days to several concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the respective control (p=<.001).

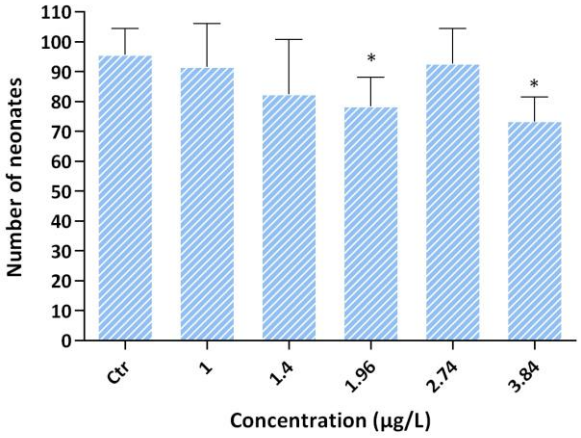

Figure 15: Average of the total number of neonates released per female of *Daphnia magna* after being exposed for 21 days to several concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the respective control (p=<0.001).

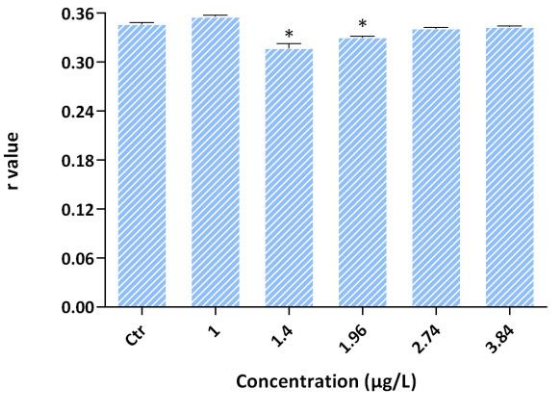

Figure 16: Average of increase rate of population growth increase (r value) for *Daphnia magna* after being exposed for 21 days to several concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the respective control (p=<0.001).

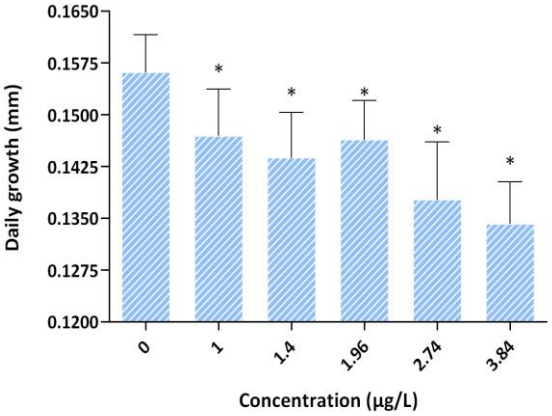

Figure 17: **Average of daily somatic growth rate of *Daphnia magna* after being exposed for 21 days to several concentrations of gold nanoparticles.** Error bars - standard deviation. * represents significant differences relatively to the respective control (p=<0.001).

Discussion

In this study, we investigated the sublethal effects of gold nanorods in tree species: the microalgae *R. subcapitata* and *C. vulgaris* and in the cladoceran *D. magna*. This type of Au-NPs induced a significant decrease in the growth of the two algae at concentrations as low as 31 µg/L. For *D. magna*, in general, significant effects were mainly observed for somatic growth rate that significantly decreased at concentrations of Au-NP as low as 1 µg/L. For reproduction, though significant effects occurred (at 1.96 and 3.84 µg/L), a dose-effect relationship was not observed with increasing Au-NP concentrations. The occurrence of significant effects at low concentrations of Au-NP where expected since other authors already reported the toxicity of this type of gold nanorods at the level of micrograms per liter. Galindo (2014) studied the effects on growth of Au-NP capped with CTAB (diameter=10 nm; length=35 nm) for *R. subcapitata* and *C. vulgaris* after 72h of exposure. He reported values of $EC_{20,72h}$ of 59.7 µg/L for *R. subcapitata* and of 95.2 µg/L for *C. vulgaris*, thus showing similar results to the ones obtained in the present study ($EC_{20,72h}$ of 39 and 79 µg/L, respectively). The two green algae appear to be less sensitive than *D. magna*. The highest sensitivity of *D. magna* to other chemicals has already been reported. For example, Blaylock et al., (1985) reported that *D. magna* was more sensitive to copper, by one to two orders of magnitude, than *Selenastrum capricornutum* (now *R. subcapitata*) and *C. vulgaris*. Garric et al., (2007) also reported the algae *Pseudokirchneriella subcapitata* (now *R. subcapitata*) to be less sensitive than *D. magna* to ivermectin, reporting values of lowest observed effect concentration of 1250 and 0.001 ng/L, respectively.

Comparing the toxicity of CTAB capped Au-NP to *D. magna* observed in this study with that reported by other works already published, it can be seen that it is similar. Galindo (2014) assessed the toxicity of CTAB capped Au-NP to *D. magna* for approximately 21 days. This author did not found significant effects in the time to release the first brood neither in the total reproduction at concentrations as high as 2.92 µg/L. In the present study, these endpoints were adversely affected by exposure to Au-NP at the concentration of 1.96 µg/L, which may due to the fact that the Au-NP studied by Galindo (2014) exhibited a slightly smaller longitudinal size. Lopes et al. (2012) also studied the toxicity of gold nanorods capped with CTAB (diameter=10 nm, length =35 nm), similar to the ones used in the present work. These authors exposed the bacterium *Vibrio fisheri* to several Au-NP concentrations for a period of 30 minutes and found EC_{20} values for the production of bioluminescence of 140 µg/L, which corroborates the high toxicity of this type

of Au-NP to aquatic biota. When comparing these results obtained with CTAB capped Au-NP with the toxicity of other Au-NP it is possible to observe that these particular CTAB capped Au-NP are, in general, more toxic. For example, Dědková et al. (2014) assessed the toxicity of Au-NP, as colloidal nanogold, to *R. subcapitata* and reported an $EC_{50,72h}$ of 14×10^6 µg/L. Botha et al. (2016) studied the effects of gold nanospheres (size= 14 nm) on the reproduction of *D. magna* after long-term exposure (14 days) and did not found significant differences comparatively to the control group until the concentration of 20000 µg/L. These differences in the toxicity of the different types of Au-NP can be associated with both the geometric configuration of the NP being tested and with the capping agents. Apparently, the mentioned studies suggest gold nanorods capped with CTAB to be more toxic than colloidal or gold nanospheres prepared in laboratory (Dědková et al., 2014; Botha et al., 2016). Actually, other researchers have already reported this. Yah (2013) and Fratoddi et al. (2015) revised the toxicity of gold nanoparticles and reported nanospheres to be less toxic to biota than nanorods. Lee et al. (2015) evaluated the effects of colloidal gold nanospheres (size between 8.5 and 12 nm) on the somatic growth of *D. magna* during 48h and they found an inhibition of growth at concentration of 5 µg/L. Again, the Au-nanorods tested in our study revealed a higher toxicity in this endpoint, since a significant reduction in the growth rate of daphnids was observed at concentrations as low as 1 µg/L, comparatively to this study with colloidal gold nanospheres.

The high toxicity of CTAB capped Au-NP may be associated with the capping agent, which has been suggested to be very toxic to biota (Rayavarapu et al., 2010; Schachter, 2013; Soenen et al., 2011). For example, Rayavarapu et al., (2010) exposed several cell lines to a concentration of 1.0 mol/L CTAB and observed 100% of cell death. As well, Takahashi et al. (2006) suggested that CTAB toxicity is much higher than other stabilizing agent, the phosphatidylcholine after comparing the toxicity of NP capped with both agents in HeLa cell line. While no effects were observed at a concentration of 0.73 mM of gold nanorods coated with phosphatidylcholine, for gold nanorods coated with CTAB 50% inhibitory effects of cell viability was found at concentration of 9.1 µM. The surfactant CTAB is also recommended for DNA extraction protocols in algae, which also evidences its potential to cause adverse effects in biological matrices (Varela-Álvarez et al., 2006; Wang et al., 2011). However, to date, no literature was found that evaluated the toxicity of the CTAB isolate at the individual level, hindering the direct comparison of the results obtained here for CTAB with other studies. In the present study, for the algae *R. subcapitata*, the value of inhibition growth was statistically different between the CTAB and the highest tested concentration of Au-NP, pointing to the possible toxic effects caused by the capping agent.

The values of EC_{20} reported in the present study for Au-NP, and according to the Commission of the European Communities (1996), these gold nanorods should be placed in the category of "extremely toxic" (EC_{20} <0.1 µg/mL). Galindo et al. (2013) also studied the effects of gold nanorods (coated with CTAB; size=35 nm) for white rot fungi species exposed until fungi achieved the maximum growth. For the most sensitive fungi, *Trametes versicolor*, the EC_{20} was 27100 µg/L, placing this Au-NP in the category "harmful". Lopes et al. (2012) evaluated the effects of gold nanorods (coated with CTAB; size=35 nm) to *Vibrio fisheri* (gram-negative bacteria) evaluated at different times during 30 minutes. For all EC_{20} measured in this work, the values were between 0.1 and 1 µg/mL, placing this Au-NP in the category "very toxic". These results suggested that toxicity differ according to the organism. Comparatively to the previously mentioned studies and to the microalgae species tested in this work, daphnids were more sensitive to Au-NP.

Conclusions

The results obtained in this work suggest that gold nanorods capped with CTAB may pose severe risks to aquatic biota (mainly producers and primary consumers), since they cause adverse effects at low concentrations. Furthermore, the obtained results allow to hypothesized that this high toxicity was mainly caused by the capping agent.

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Chapter 3
Multigenerational effects of gold nanorods to freshwater microalgae

Abstract

The exponential increase in the use of nanoparticles over the past years, without an accurate understanding of their effects on the environment, has caused concern to the ecotoxicologists. The aim of this study was to evaluate the generational effect of sublethal levels of gold nanorods (Au-NP) in the growth rate of two microalgae. The green algae *Chlorella vulgaris* and *Raphidocelis subcapitata* was exposed to the corresponding $EC_{10,72h}$ for growth for four generations and they were exposed to serial dilutions of Au-NP to evaluate effects on growth. To assess generational effects, each algae was exposed to the corresponding $EC_{10,72h}$ for growth for four generations. The sublethal sensitivity of each generation to Au-NP was assessed. For *C. vulgaris*, an increase on its tolerance to Au-NP was observed at the third generation since no significant effects on growth were observed between control and Au-NP treatments from this generation onwards. As for *R. subcapitata*, overall, the generational exposure to Au-NP increased its sensitivity to this NP. These results suggested that long-term effects should be included in ecological risk assessments since standard toxicity may either over or underestimate the risk.

Keywords: multigenerational, *Chlorella vulgaris*, *Raphidocelis subcapitata*, gold nanorods, CTAB

Introduction

Nanomaterials (NMs) exist in the environment originated either from natural or artificial sources (Rosenkranz, 2010). However, in the last decades the unique characteristics of NMs started to be exploited by the industry and its production and applications experienced a boom. Although NMs bring many important benefits to the society, such as in areas of diagnosis of diseases, their unique characteristics have been associated with several adverse effects to biota (Farkas et al., 2010; Larguinho et al., 2014; Rosenkranz, 2010; Van Hoecke et al., 2008). Among the wide diversity of NM being produced, gold nanoparticles (Au-NP) have been increasingly used in the society due to the fact that their optical and electric properties may be easily tunable by changing their size, shape or surface chemistry (Barkalina et al., 2014). These characteristics make Au-NP attractive for different areas, namely for medicine that has mainly explored these NMs to be used in diagnostic procedures (e.g. markers for tumor cells). Though these beneficial applications of Au-NP to the society, some works have already identified several adverse effects that these NP may pose to biota, and that the severity of such observed toxic effects is not only influenced by the Au-NP concentration but also by other characteristics intrinsic to the NP (e.g. its geometric configuration, size, surface charge, chemical composition of the capping/stabilizing agents) (Nasser et al., 2016; Wan et al., 2015; Yah, 2013; Zhang et al., 2012). Bozich et al. (2014) studied the effects of Au-NP bearing different stabilizing agents (trisodium citrate-Cit, poly(allylamine) hydrochloride-PAH, mercaptopropionic acid and trimethylammonium bromide-CTAB) in *Daphnia magna*. After exposure to lethal and sublethal concentrations (below 50 µg/mL) of the Au-NPs these authors reported that positively-charged particles (with PAH and CTAB) were more toxic and exhibited a lower aggregation rate than the negatively-charged ones. Bozich et al. (2014) suggested that these results were related with the fact that positively charged Au-NP had a higher affinity to the negatively charged cellular membranes. Browning et al., (2009, 2013) exposed zebrafish embryos to Au-NPs with different sizes (11.6±0.9 nm and 86.2±10.8 nm) and observed that the smaller caused higher mortality and deformities in the embryos.

Though some works on the toxic effects of Au-NP already exist, most are focused on the effects in cell lines and small mammals in order to assess their biocompatibility (Conde et al., 2012; Moretti et al., 2013; Rayavarapu et al., 2010). Studies on their effects in aquatic biota are scarce and most of them use standardized approaches that, though being important for first stage risk assessment, lack some ecological relevancy and neglect potential adverse effects that may

appear or disappear across generations. Recently, some works started to focus on the long-term effects of Au-NP in biota trying to increase the ecological relevancy of the generated ecotoxicity data, but they are focused only in one model organism, the nematode *Caenorhabditis elegans*. Kim et al., 2013exposed *C. elegans* to Au-NP by feeding them with contaminated food (with $5x10^{10}$, $25x10^{10}$ and $50x10^{10}$ particles of Au-NP/mL) items through four generations. Independently of the Au-NP concentration, the authors observed no significant changes in lethal sensitivity to Au-NP across generations. However, for reproduction and abnormalities in the reproductive system, the authors observed that at F2 these parameters were more affected than in F0, but organisms were able to recover in F3 and F4. More recently, Moon et al. (2017) investigated the multigenerational effects of Au-NP (colloids; size between 8.5 and 12 nm) on *C. elegans* after continuous or intermittent supply with food items contaminated with Au-NP. Intermittent exposure to Au-NP caused a decrease in F3 reproduction, comparatively to control, of 63%, 53%, and 40%, at concentrations of 5×10^{10}, 25×10^{10}, and 50×10^{10} particles Au-NP/mL, respectively. In continuous exposure, an increase in the total abnormalities rate were observed for F1 (13.3%), F2 (15%), F3 (17.5%) and F4 (23.3%) generations of *C. elegans* exposed at 50×10^{10} particles/mL. This study showed the effects of multigenerational assay may vary according to different exposure patterns, exposure levels, and recovery periods.

To the present, there is no information on the effects of multigenerational exposure to Au-NP on freshwater organisms. Therefore, the aim of this study was to evaluate the multigenerational effects of gold nanorods in two microalgae, *Chlorella vulgaris* and *Raphidocelis subcapitata*.

Materials and methods

Tested substance

Gold nanorods (Au-NP) capped with the surfactant cetyltrimethylammonium bromide (CTAB) (A12-10-780) were purchase to Nanopartz™ (Salt Lake City, UT, USA) as dispersion in deionized water with less than 10mM of CTAB. The concentration of the Au-NP in the dispersion was 35 μg/ml and their primary sizes were 10 nm for diameter and 38 nm for length (information

specified by Nanopartz™, 2006). The surfactant hexadecyltrimethylammonium bromide (CTAB) 95% was supplied by Sigma-Aldrich® as a powder.

Gold nanorods characterization

The physical characterization of the stock Au-NP suspension, the highest tested concentration for each alga (90 µg/L for *C. vulgaris* and 53 µg/L for *R. subcapitata*), and the concentrations causing 10 % of growth inhibition in each algae (21 µg/L for *C. vulgaris* and 22 µg/L for *R. subcapitata*) was carried out. This characterization was performed at time 0h (just after the preparation of the suspension) and after 72h (corresponding to the duration of the toxicity assays). During this period of time the suspensions were maintained under the same exact conditions as those of the ecotoxicity assays that were carried out.

Ultraviolet/visible/infrared (UV/Vis/IR) spectroscopy was used to determine the spectra of light that is absorbed and scattered by the Au-NP dispersions (NanoComposix, 2012). This allowed determining the absorbance peak of the stock dispersion (to compare with the value provided by Nanopartz™) and of the Au-NP concentrations in the assay medium.

The surface charge and the zeta potential of the Au-NP in dispersions were determined through electrophoretic light scattering (ELS) in a Zetasizer Nano ZS with Zetasizer Software (Malvern ZetaSizer, 2013). This technique was performed in a disposable folded capillary cell (Figure 18).

Figure 18: Disposable folded capillary cell for measurement of zeta potential (source: LabBulletin, 2009)

The hydrodynamic diameter was measured by dynamic light scattering (DLS), as well in a Zetasizer Nano ZS with Zetasizer Software, (Malvern ZetaSizer, 2013), to estimate size. Size was also characterized by using Transmission Electronic Microscopy (TEM) (Philips CM100). For this, 2mL of stock solution were centrifuged at 10630 rpm during 30 minutes to separate CTAB from Au-NP. After this, the supernatant was discarded and 0.5mL of Milli-Q water were added and the solution was resuspended. One drop of centrifuged solution was placed on a carbon coated copper grid and left to dry for 24h. The values of EC_{10} for both microalgae were similar (EC_{10}=21 and 22 µg/L), so, the results of characterization of EC_{10} solution are the same for both microalgae (22 µg/L).

Test species

The effects of multigenerational exposure to Au-NP on two species of microalgae: *Raphidocelis subcapitata* and *Chlorella vulgaris*, were studied. The cultures of *R. subcapitata* and *C. vulgaris* were maintained at the same conditions as those described in Chapter 2 in Woods Hole MLB culture medium (Stein, 1973; please see section Materials and Methods – Test species). The MBL medium, as all material used to prepare cultures and ecotoxicological assays, was previously sterilized in an autoclaved at 121°C at 1 Bar, for 30 minutes.

Generational exposure of microalgae to gold nanorods

The two species of microalgae were exposed to the respective Au-NP concentration causing 20% of reduction in growth rate after 72h of exposure ($EC_{20,72h}$, computed in Chapter 2). The $EC_{20,72h}$ was selected because it is considered the threshold for effect. However, after several attempts, it was observed that after being exposed for one generation, to the respective $EC_{20,72h}$, the two microalgae were not capable to attain the log growth phase, which impaired the continuity of the experiment. Therefore, generational exposure was afterwards performed at the concentration causing 10% of growth inhibition ($EC_{10,72h}$) after 72h of exposure. Accordingly, both algae were exposed for four generations, from F1 to F4, to the respective $EC_{10,72h}$ of Au-NP. Inoculation from one generation to the other was made with inoculates at exponential growth phase (Figure 19). All generations of each alga were maintained under the same conditions, at 20±1ºC with continuous cool-white fluorescent light (fluorescent light tubes: OsramL36W/10; 100 $\mu E/m^2/s$).

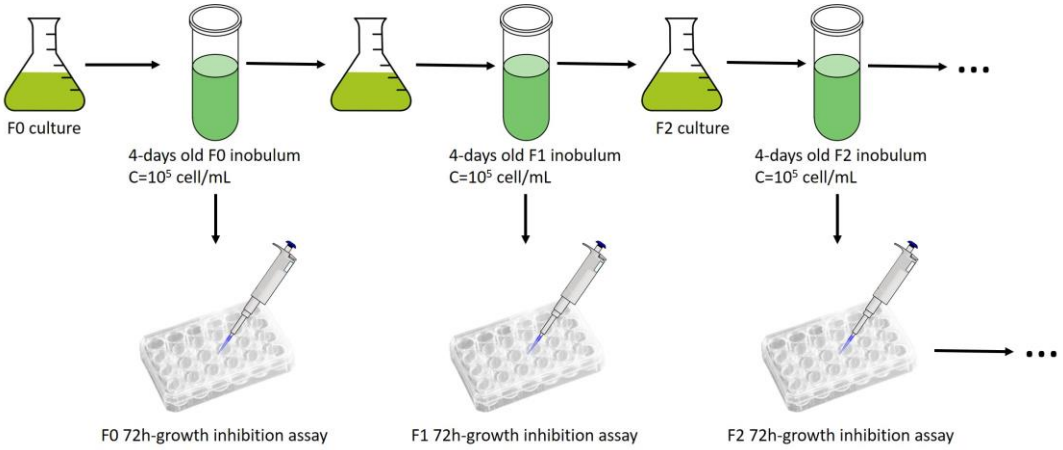

Figure 19: Scheme illustrating the sequential exposure of generations of *Raphidocelis subcapitata* and *Chlorella vulgaris* to the respective $EC_{10,72h}$ for growth rate. C=algae concentration.

72h-growth inhibition assay

The 72h-growth inhibition assays was performed with all the generations of each microalgae and followed the OECD Guideline 201 (OECD, 2004). All generations of *Raphidocelis subcapitata* and *C. vulgaris* were exposed to the same concentrations of Au-NP and of CTAB as F0 generation in Chapter 2. All the assays were conducted in 24-wells microplates at 23±1°C under continuous light of 4000lx, using the same experimental design as that described in Chapter 2 (please see section 72h-growth inhibition assays with microalgae - Chapter 2).

At the end of 72h, the absorbance (ABS) was measured at 440 nm in a spectrophotometer (Jenway, 6505 UV/Vis) and converted in cell density per volume according to the following equations:

$$Cell/mL = -17107.5 + \mathrm{Abs440} \times 7925350$$ (*R. subcapitata;* r^2=0.98; p≤0.05)

$$Cell/mL = -155820 + \mathrm{Abs440} \times 13144324$$ (*C. vulgaris;* r^2=0.91; p≤0.05)

The population growth rate (r) was also calculated, according to the following equation:

$$r = \frac{\ln N_F - \ln N_I}{t}$$

where N_F is the mean number of algae in the end of test (cell/mL), N_I is the mean number of algae in the start of the test (cell/mL) and t is the time of exposure (days)

The percentage of population growth inhibition was also calculated, by using the equation:

$$I(\%) = ((N_C - N_{con})/N_C) \times 100$$

where N_C is the mean number of algae in control and N_{con} is the mean number of algae in respective concentration.

Data analysis

The effective concentration causing 10% of growth inhibition was computed for each generation of each algae by fitting a logistic model with STATISTICA 8.0 software™ (Zar, 1999). After confirmed the ANOVA assumptions (Kolmogorov–Smirnov test for normality of data, and Bartlett's test for homoscedasticity of variance), a two-way ANOVA analysis of variance was performed to determine if there were significant differences among treatments and interaction between factors. The two-way ANOVA was followed by the multicomparison Holm-Sidak test.

Results

Gold nanorods characterization

The zeta potential of the stock solution, was 71.6 mV, the conductivity 0.176 mS/cm, the hydrodynamic diameter 30.81 nm and the polydispersity index was 1.0 (Table 1; Fig. 21). The value for absorbance of stock solution had a principal absorption peak at 780 nm confirming the value provided by the company (Nanopartz™, 2006) (Figure 20).

For the others solutions (prepared with MBL medium) the values of conductivity did not change much, ranging from 0.461 to 0.501 mS/cm (Table 1). As for the zeta potential, the values at 0h and 72h, for the EC_{10} and the highest tested concentrations (53 and 90 µg/L) were similar being within the range of low stability. The surface charge of the Au-NPs was negative with the

exception of concentration 90 µg/L, at 0h, where they exhibited a positive surface charge (Table 1). The hydrodynamic size of the $EC_{10,72h}$ and of the two highest concentrations of Au-NP was much higher than the primary size, always higher than 631.9 nm (Table1). Furthermore, the hydrodynamic diameter increased with concentrations, being higher at 90 µg/L. Differences in this parameter were also observed between dispersions with 0h and 72h, being at least 2-fold higher at 72h comparatively to 0h (Table 1). The polydispersity index (ranging from 0 to 1) was high for the three characterized Au-NP concentrations, being always higher than 0.573 (Table 1).

Table 1: Physical characterization of Au-NP dispersions.

	EC_{10} (22 µg/L) (both microalgae)		**53 µg/L (*Raphidocelis subcapitata*)**		**90 µg/L (*Chlorella vulgaris*)**		**Stock solution (35 000 µg/L)**	
	0h	**72h**	**0h**	**72h**	**0h**	**72h**	**Supplier information**	**Measured in this study**
Conductivity (mS/cm)	0.462	0.492	0.501	0.461	0.480	0.471	-	0.176
Zeta Potential (mV)	-6.63	-14.1	-6.68	-12.4	6.45	-11.0	≈0	71.6
Diameter size (nm)	631.9	1834	672.8	1121	714.2	2323	10	30.81
Polydispersity index	0.741	0.922	0.596	0.799	0.573	0.589	-	1.0

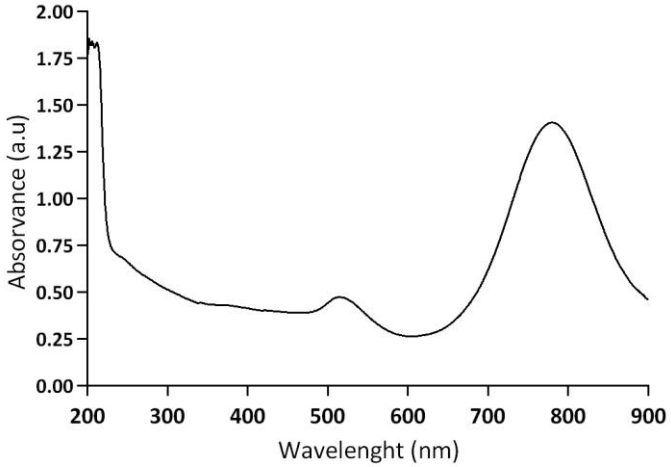

Figure 20: Spectra of light absorbance of the stock solution of Au-NP.

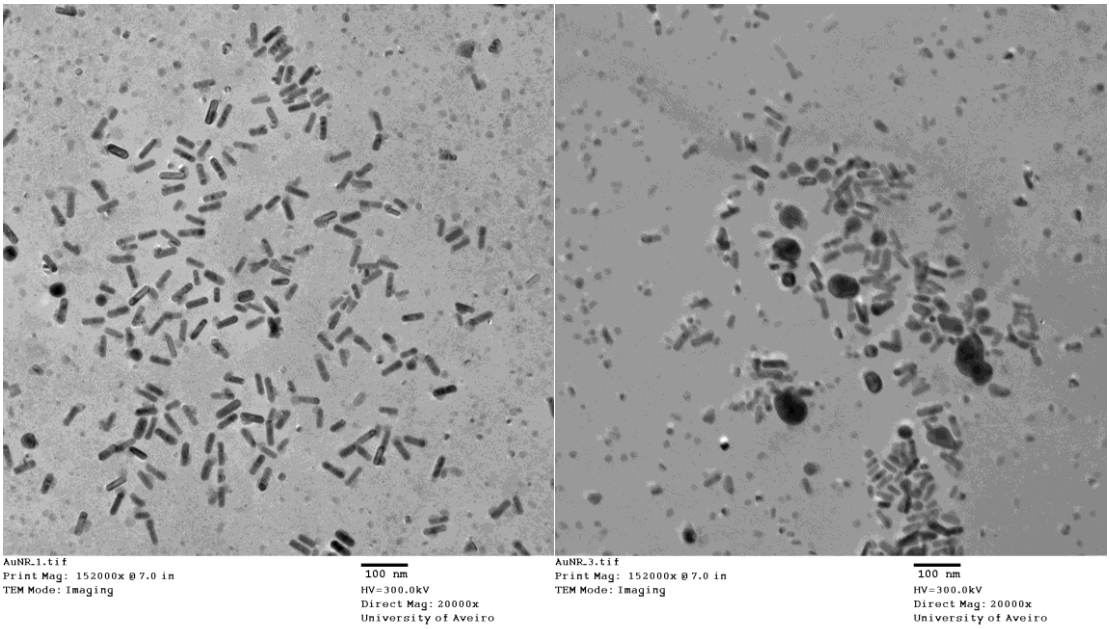

Figure 21: Transmission-electron microscope images of gold nanorods of the stock solution (20 000x)**.**

72h-growth inhibition assay

Concentrations of Au-NP above 24 µg/L induced a significant decrease in the growth rate of *C. vulgaris* in F0, comparatively to the control (Figure 22; Holm-Sidak: p=<0.001). The generational exposure of this algae to the $EC_{10,72h}$, tended to decrease the effects caused by the Au-NP on its population growth rate relatively to the respective control. After exposure for just one generation to the $EC_{10,72h}$ (F1), a significant decrease in growth rate, comparatively to the respective control, was only observed for Au-NP concentrations of 40, 53 and 90 µg/L (Figure 23; Holm-Sidak: p=<0.001). And at F2, similar results to F1 were obtained (Figure 24; Holm-Sidak: p=<0.001). The concentrations of Au-NP causing 10% of effect in growth rate of *C. vulgaris* were: 21 µg/L (95% CL: 10-31 µg/L) for F0, 46 µg/L (95% CL: 16-76 µg/L) for F1 and 86 µg/L (95% CL: 49-123 µg/L) for F2. For generations F3 and F4, no significant differences were observed in growth rate of *C. vulgaris* exposed to the control and to all tested Au-NP concentrations (Figure 25). and Figure 26; Holm-Sidak: p=0.135 to F3 and p=0.051 for F4). Furthermore, growth rate of *C. vulgaris* in the control was higher in F0 comparatively to F1, F2 and F4.

Moreover, a significant decrease in growth rate, when exposed under controlled conditions, was observed between generations F1, F2 and F4 and generation F0 (Holm-Sidak: p=<0.001).

Exposure to 0.257 mM of CTAB, induced a decrease in growth rate of *C. vulgaris* comparatively to the control, for all tested generations (Table 2; Holm-Sidak test: p=<0.001).

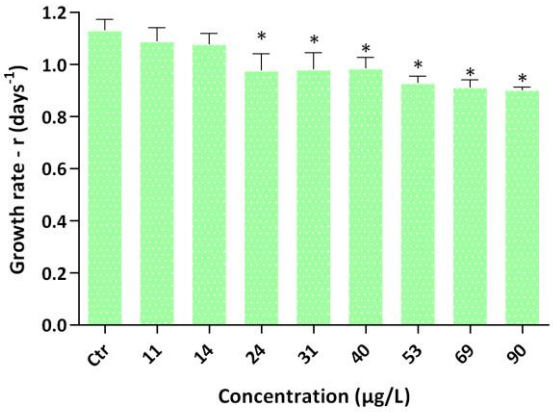

Figure 22: Average of daily growth rate for F0 of *Chlorella vulgaris* after being exposed, for 72h, to a range of concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the control (p=<0.001).

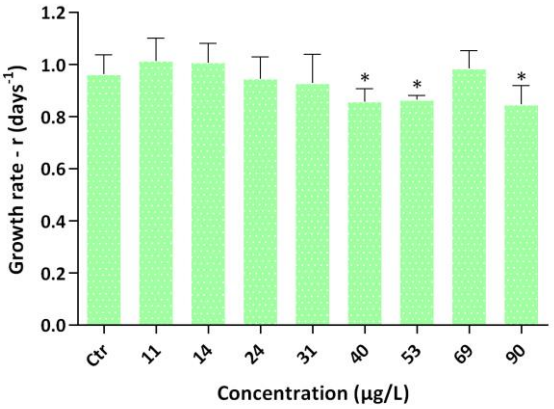

Figure 23: Average of daily growth rate for F1 of *Chlorella vulgaris* after being exposed, for 72h, to a range of concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the control (p=<0.001)

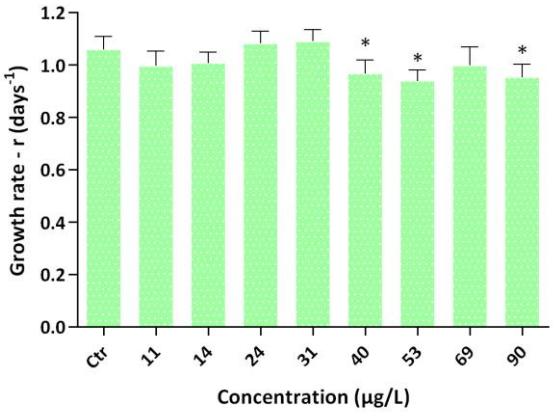

Figure 24: Average of daily growth rate for F2 of *Chlorella vulgaris* after being exposed, for 72h, to a range of concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the control (p=<0.001).

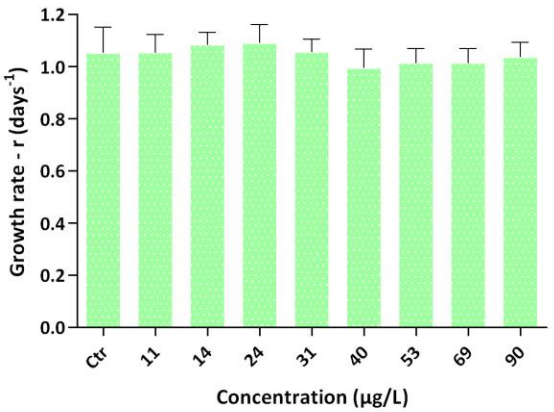

Figure 25: Average of daily growth rate for F3 of *Chlorella vulgaris* after being exposed, for 72h, to a range of concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the control (p=0.264).

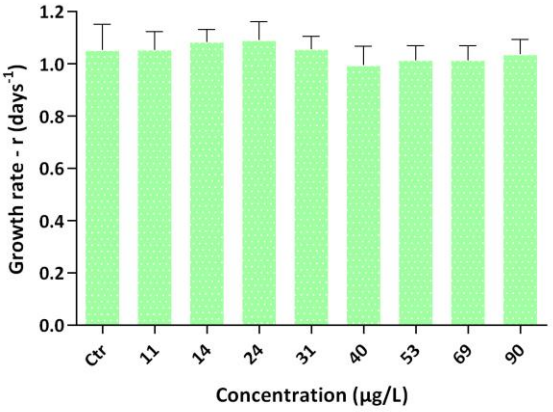

Figure 26: Average of daily growth rate for F4 of *Chlorella vulgaris* after being exposed, for 72h, to a range of concentrations of gold nanoparticles. Error bars - with respective standard deviation. * represents significant differences relatively to the control (p=0.070).

Table 2: Average of the percentage of growth inhibition relatively to the control (and standard deviation within parenthesis) of *Chlorella vulgaris* exposed, for 72h, to CTAB.

	% of growth inhibition	P value
F0	15.5 (±6.9)%	<0.001
F1	9.6 (±9.6)%	0.024
F2	16.7 (±5.1)%	<0.001
F3	36.3 (±19)%	<0.001
F4	19.0 (±4.8)%	<0.001

Concentrations of Au-NP above 40 µg/L induced a significant decrease in the growth rate of *R. subcapitata* in F0, comparatively to the control (Figure 27; Holm-Sidak: p=<0.001). After one generation (F1) of exposure to the $EC_{10,72h}$ the sensitivity of *R. subcapitata* to Au-NP increased, since significant effects in growth rate, comparatively to the control, were observed at concentrations equal or higher than 14 µg/L (Figure 28; Holm-Sidak: p=<0.001). At F2, a significant decrease, comparatively to control, in the growth rate was observed at concentrations of Au-NP above 24 µg/L concentration (Figure 29; Holm-Sidak, p=<0.001). In this generation, it was visible a much larger decrease in the growth rate for higher concentrations, comparatively to the F0 and F1 generations (at 53 µg/L: F0=29.5%, F1=24.6% and F2=75.8%). Significant differences were observed in growth rate between F3 and the other generations for concentrations of 11, 14, 40 and 53 µg/L (Figure 30; Holm-Sidak: p=0.048). However, in F4, significant reduction in growth rate, relatively to the control, occur again at Au-NP concentrations above 14 µg/L (Figure 31;

Holm-Sidak: p=<0,001). The concentrations of Au-NP causing 10% of effect in growth rate of *R. subcapitata* were: 22 µg/L (95% CL: 13-33 µg/L) for F0, 8 µg/L (95% CL: 2-13 µg/L) for F1, 9 µg/L (95% CL: 3-15 µg/L) for F2, 14 µg/L (95% CL: -46-67 µg/L) for F3, 9 µg/L (95% CL: 4-13 µg/L) for F4 (EC20 were also computed and are shown in Table 4 – Annex 1). Moreover, significant decrease in growth rate, when exposed under control conditions, was observed between generations F2 and F4 and generation F0 (Holm-Sidak: p<0.001).).

Exposure to 0.152 mM of CTAB, caused a significant decrease in growth rate in all generations except for F3 (Table 3 Holm-Sidak test: p<0.001).

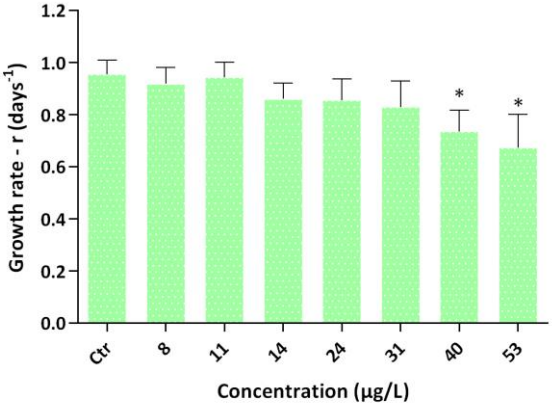

Figure 27: Average of daily growth rate for F0 of *Raphidocelis subcapitata* after being exposed, for 72h, to a range of concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the control (p=<0.001).

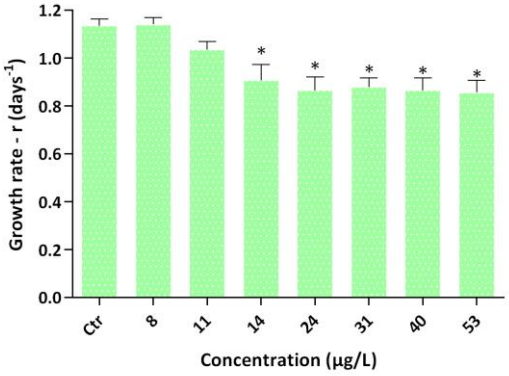

Figure 28: Average of daily growth rate for F1 of *Raphidocelis subcapitata* after being exposed, for 72h, to a range of concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the control (p=<0.001).

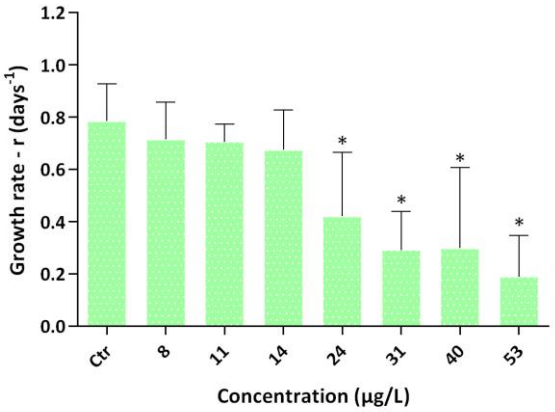

Figure 29: Average of daily growth rate for F2 of *Raphidocelis subcapitata* after being exposed, for 72h, to a range of concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the control (p=<0.001)).

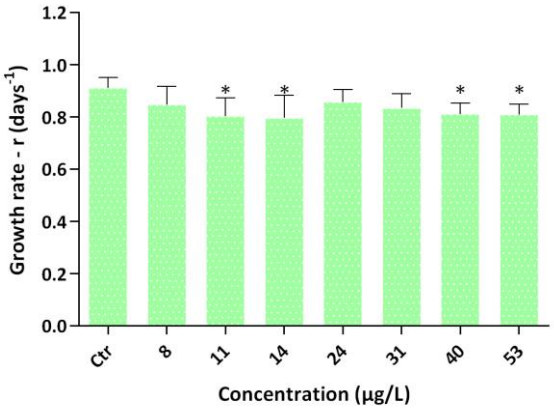

Figure 30: Average of daily growth rate for F3 of *Raphidocelis subcapitata* after being exposed, for 72h, to a range of concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the control (p=0.0048).

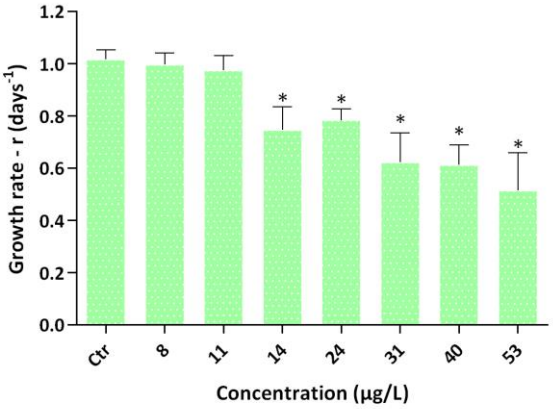

Figure 31: Average of daily growth rate for F4 of *Raphidocelis subcapitata* after being exposed, for 72h, to a range of concentrations of gold nanoparticles. Error bars - standard deviation. * represents significant differences relatively to the control (p=<0.001).

Table 3: Average of the percentage of growth inhibition relatively to the control (and standard deviation within parenthesis) of *Raphidocelis subcapitata* exposed for 72h to CTAB.

	% of growth inhibition	P value
F0	15.5 (±10.4)%	0.012
F1	33.3 (±6.50)%	<0.001
F2	41.9 (±25.0)%	=0.001
F3	6.9 (±6.90)%	=0.076
F4	31.2 (±16.1)%	=<0.001

Discussion

All the concentrations of Au-NPs tested in MBL medium showed a high level of aggregation at time 0h and this aggregation increased with time, being higher at 72h of exposure. This is corroborated by the increase in size that was observed in the tested Au-NP concentrations comparatively to the stock solution. One more indication that aggregation occurred was the values of zeta potential remained in a range considered typically unstable (-30 mV to +30 mV) (Rogers et al., 2010). These results were expected, because MBL medium has a high ionic strength. In these conditions the electrical double layer at the NPs surface may become thinner, with a decrease in the zeta potential, thus, lowering the repulsion between the NPs in suspension and increasing the probability of aggregation. Lopes et al., 2012 tested the diameter values by DLS of gold nanorods in two different mediums: Milli-Q water (low ionic strength) and ASTM (high ionic strength) and they observed an increase in size from 52 to 308 nm, respectively. Furthermore, the results here obtained shown that time influenced the aggregation of the NPs. The size of Au-NPs almost doubled from time 0h to 72h. Other authors have already observed this time-dependency in size of Au-NP aggregates. Afrooz et al. (2014) reported a gradual increase in the size of gold nanopsheres over time. These authors also observed that within a 6h period aggregates attained a critical size that acted as a critical nucleating size for fast growing aggregates. Afrooz et al. (2014) suggested that the low electrokinetic energy barrier of Au-NP promoted a short-range interaction between the NP, promoting the occurrence of a process similar to the aggregative nucleation, where particles or aggregates are forced to join already existing aggregates. Since, the zeta potential of the here tested Au-NP was low it is hypothesised that a similar processes could have occurred over time, increasing the size of the aggregates from time 0h to 72h.

Raphidocelis subcapitata showed higher sensitivity to the Au-NP coated with CTAB than *C. vulgaris*. In addition to this higher sensitivity to Au-NP, overall *R. subcapitata* became more sensitive to the Au-NPs after generational exposure, while *C. vulgaris* was able to acquire an increased tolerance to this NP. The different responses of the two algae may be related to the different surface area:volume ratios. Based on the size of the two microalgae (given by Nygaard et al. (1986); Geiger, 2014) and in the formulas to compute biovolumes and surface areas (Sun & Liu, 2003) the ratio surface area:volume computed for *R. subcapitata* ranges approximately from 5.83 to 199, being higher than those computed for *C. vulgaris* (between 1.5 and 6). This is inline with data reported in scientific literature that states that cells with large surface area to volume ratios

tend to be more sensitive than those with smaller ratios (Levy et al., 2007). Furthermore, *R. subcapitata* by exhibiting a higher surface area relatively to *C. vulgaris* could exhibit more binding sites and receptor–ligand interactions at the cell membrane level promoting a higher biding of Au-NP to the cell membrane and a higher internalization of Au-NP (Schwab et al., 2011; Ma and Li, 2013).

Regarding the effects of the Au-NP after generational exposure, as mentioned above, different outputs were observed for the two algae. While *C. vulgaris* was capable of acquiring an increased tolerance to this chemical, *R. subcapitata*, exhibit some variability throughout the generations, but mainly its sensitivity increased. This consistent higher sensitivity of *R. subcapitata* relatively to *C. vulgaris* to Au-NP could be associated with the fact that *C. vulgaris* tend to form aggregates more easily than *R. subcapitata* (Environmental Protection Series, 2007; Fisher et al. 2016). These aggregates may reduce the exposure of inner cells of the algae-aggregate to the Au-NP, thus, reducing toxicity. Furthermore, the fact that *R. subcapitata* exhibits a higher surface area:volume ratio comparatively to that of *C. vulgaris* could also explain the higher sensitivity of the former species, since in the literature has been reported that cells with large surface area to volume ratios tend to be more sensitive than those with smaller ratios (Levy et al., 2007). Furthermore, *R. subcapitata* by exhibiting a higher surface area relatively to *C. vulgaris* could exhibit more binding sites and receptor–ligand interactions at the cell membrane level promoting a higher biding of Au-NP to the cell membrane and a higher internalization of Au-NP (Schwab et al., 2011; Ma and Li, 2013). None of the algae acquired an increased tolerance to the capping agent CTAB, though generational exposure to Au-NP involved the presence of Au and of CTAB. It is suggested that CTAB is mostly bond to Au-NP and probably the increased tolerance to the Au-NP is related with tolerance to the ions dissociating from the NP.. The generational effects of nanoparticles, namely Au-NP, has not been much studied, and no studies have been published with microalgae. However, some studies published with other species report both the maintenance and the increase of sensitivity to NPs. Moon et al. (2017) evaluated the multigenerational effects of Au-NP (colloids; size between 8.5 and 12 nm) on *Caenorhabditis elegans* after continuous or intermittent exposure through Au-NP contaminated food items. Both continuous and intermittent generational exposure to Au-NP caused adverse effects in reproduction. However, continuous exposure affected reproduction from F2 to F4 while intermittent exposure caused more pronounced effects on F3. But, in both types of exposure after four generations, *C. elegans* did not acquired and increased tolerance to Au-NP exposure. Ma et al. (2016) studied the effects on plant growth and the oxidative stress after multi-

generational cerium oxide nanoparticles (spherical CeO_2-NP; size=20 nm) exposure over a range of concentrations (0–1000 mg/L) on the terrestrial plant *Brassica rapa*, during 3 generations (F0, F1 and F2). Multigenerational exposure to CeO_2-NP caused a significant reduction of seed yield and seed quality, which is critical for continued food security. Multigenerational exposure to CeO_2-NPs altered the plant physiological and biochemical responses in subsequent generations of plants, and caused greater reductions in plant growth and development and an increased on the ROS production, i.e. increased the sensitivity of the plants to the NP.

Though no studies on the multigenerational effects of Au-NP exist for microalgae, the effects that generational exposure of this group of organisms to some chemicals (including metals) has already been reported, and no clear pattern of increasing sensitivity or tolerance to the chemical can be identified. For example, Muyssen and Janssen (2001) exposed *R. subcapitata* and in *C. vulgaris*, to a concentration of 65µg/L of zinc and observed if these algae were capable to acquire an increased tolerance to this metal comparatively to a F0 (not exposed to increased zinc concentrations). They observed that both algae species acquired and increased tolerance to zinc through physiological acclimation. However, Stachowski-Haberkorn et al. (2013) evaluate the capacity of microalgae *Tetraselmis suecica* to develop long-term tolerance to the herbicide diuron. During the first 25 generations this alga exhibited a high sensitivity to this pesticide, by exhibiting a 2 to 2.5-fold increase in the doubling-time of growth when exposed to the pesticide. But, after being exposed 25 to 32 generations of exposure to of 5 µg/L to diuron, *T. suecica* was able to tolerate the pesticide, showing doubling-time of growth similar to that in the control.

Conclusions

The results obtained in the present work reveal that long-term effects of Au-NP are species dependent. The standard 72h growth inhibition assay revealed to over- (for *C. vulgaris*) or underestimate (for *R. subcapitata*) the long-term effects that Au-NP may pose to microalgae, highlighting the need to study the long-term effects of NPs to different taxonomic groups in order to increase the accuracy of ecological risk assessment.

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Chapter 4
Conclusions and prospective work

Conclusions and major findings

The results obtained in Chapter 2 revealed that the gold nanorods capped with CTAB are extremely toxic to aquatic biota, accordingly with the classification of the European Commission (1996). The assays carried out with the three model species (*C. vulgaris*, *R. subcapitata* and *D. magna*) demonstrated the occurrence of significant effects at micrograms per liter of Au-NP. For *D. magna*, significant effects in somatic growth were observed at concentrations as low as 1 µg/L. At present, there are any works, published in the scientific literature, describing measured environmental concentrations of Au-NP, but, probabilistic models estimate that these concentrations will be very low (Mahapatra et al., 2015). Mahapatra et al. (2015) modeled the Au-NP and predicted their environmental concentrations, by using information on the maximal consumption of Au-NP from medical applications in the United Kingdom and United States. These authors estimated concentrations of Au-NP (originated only from medical use) in surface waters of 468 and 4.7 pg L^{-1}, respectively for the UK and US. Therefore, the toxicity results obtained in the present work for the microalgae and cladocera suggest that Au-NP will not constitute a risk for freshwater biota. Of course, it must be considered that this model lacks the input of other sources of Au-NP and it does not take into consideration the increasing use of Au-NP in the future, which, may in the future change the evaluation of the risk of these NP.

The cladoceran *D. magna* revealed to be the most sensitive species to the gold nanorods, comparatively to the two tested species of microalgae and to other species studied in other works where gold nanorods, similar to the ones used here, were tested (Lopes et. al., 2013; Galindo, 2014). This highlights the need to assess the toxicity of Au-NP to several taxonomic groups and as well assess different sublethal endpoints in each group. In this particular case of the gold nanorods, the inclusion of the results obtained for somatic growth with *D. magna*, to the dataset obtained with the microalgae and from other published works, led to the classification of the Au-NP to the category of "extremely toxic" (EC, 1996). Furthermore, the obtained results suggested that the high observed toxicity was associated not only to the Au-NP but as well to its capping agent CTAB. Though significant differences in the toxicity of CTAB and the corresponding tested Au-NP concentrations (having the same CTAB concentration) were only observed for the algae *R. subcapitata*, the percentage of growth inhibition of the two algae was slightly higher when exposed to the Au-NP (*C. vulgaris*: 15.5% vs 20% and *R. subcapitata*: 15.5% vs 29%, for CTAB vs Au-NP, respectively), suggesting the effect induced by the Au-NP in addition to that induced by

CTAB. These results highlight the care to be taken when selecting the capping agent to stabilize this type of Au-NP. The surfactant CTAB is indeed widely used to produce Au nanorods because it presents several advantages that makes it more efficient, namely CTAB forms a lipid bilayer with a positive surface charge that strongly adsorbs to the gold nanorod surface (Rayavarapu et al., 2010). This bilayer is very important in the formation of rod-shaped morphology, since it restricts the normal growth of the NP in on direction. However, its use should be thought due to its high toxicity to biota and other surrogates should be explored.

In chapter 3, the highest sensitivity of *R. subcapitata* to the Au-NP, relatively to *C. vulgaris*, was corroborated even after the generational exposure to the NP. While *C. vulgaris* was able to acquire an increased tolerance to the Au-NP (significant reduction of population growth between control and Au-NP disappeared after exposure to the Au-NP for three generations), *R. subcapitata*, in general, showed an equal or increased sensitivity to this NP, after both algae being exposed for four generations to the corresponding $EC_{10,72h}$. Interestingly, none of the algae acquired an increased tolerance or sensitivity to the capping agent CTAB. Probably this occurred because, during generational exposure to Au-NP, most of the CTAB was bound to the NP and, therefore, the algae were not exposed to the CTAB isolated. For the Au-NP, most probably the highest tolerance shown by *C. vulgaris* was related with and increased tolerance to Au dissociated from the NP. These results, obtained after generational exposure to Au-NP, emphasize the need to include the assessment of long-term effects of Au-NP in ecological risk assessment studies, since the use of standard protocols may lead to both over or underestimation of risk.

Prospective work

Currently, only few studies have investigated the long-term effects of Au-NPs to freshwater biota (Baun et al., 2008). Accordingly, one of the major goals of the present work was to contribute to decrease this knowledge gaps by generating information on the long-term effects that gold nanorods may cause in microalgae (that as producers are at the base of the trophic chain). However, further knowledge is still needed in order to allow a more accurate extrapolation of the effects that Au-NP may pose to freshwater ecosystems and, consequently, improve conservation actions. Following the study developed within this thesis and the identified knowledge gaps, it would be interesting to:

(i) further assess the long-term effects of Au-NP for longer periods of time, involving the testing of more generations. This topic follows the results obtained with generational exposure of *R. subcapitata* Au-NP. This alga exhibited some variability in the generational sensitivity to Au-NP, exhibiting higher or lower sensitivity to Au-NP comparatively to F0. It is important to understand if this pattern will prevail over generations or if after several generations the sensitivity of the algae to the NP stabilizes (either as becoming more or less sensitive)

(ii) determine long-term effects of Au-NP to other freshwater biota, namely invertebrates representative of different trophic levels (primary, secondary consumers). Namely, the results obtained in the present work indicate *Daphnia magna* as an interesting model species. It exhibited a higher sensitivity to the tested Au-NP comparatively to the algae, therefore is important to assess if this higher sensitivity persists or increases (worst case-scenario) after generational exposure to the Au-NP or if it becomes more tolerant (as observed for the alga *C. vulgaris*). To follow up the assays run in chapter 2 with this species, it will be needed to find a concentration that would cause effect on reproduction after a generational exposure to Au-NP, in order to allow proceeding with the generational assays. We found effects on growth at the concentrations tested, but it would be important at the population level to try to understand if there may be a decrease in the number of the species at long-term.

(iii) Within the framework of a changing world (e.g. involving climate changes, pop-up of new industries/activities that release new chemicals into de environment), long-term studies should also involve exposure to the Au-NP in mixture with other environmental perturbations (e.g. other chemicals) in order to simulate more real and ecologically relevant scenarios of exposure. Galindo (2013) already studied the influence of temperature, hardness and humic substances in the toxicity of gold nanorods to cladocerans. However, his studies did not involve long-term exposures.

(iv) Inorganic elements may accumulate in the biota, and, for in the case of some elements, biomagnification may occur. These processes are especially relevant and may be potentiated if exposure occurs for a long-term, even if it occurs at very low levels (as expected for NPs). Simple food web may be simulated in laboratory experiments to understand these processes. For example, a simple lab-food web could be constituted by the species studied in this study and by hydra (predatory

Daphnia magna): micoalgae (producer) – cladocera (primary consumer) – cnidaria (secondary consumer). (Figure 32).

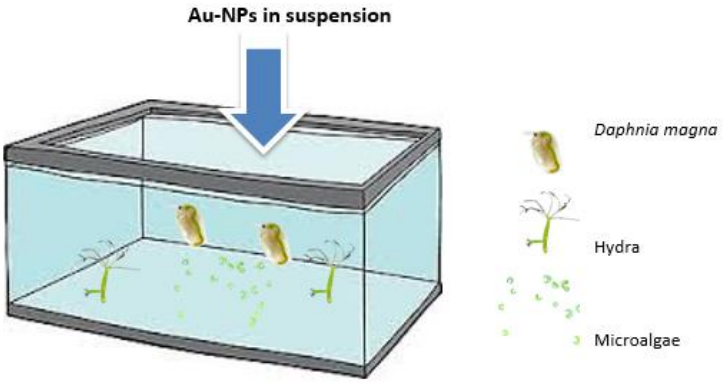

Figure 32: Illustration of a possible experimental set up to assess biomagnification of Au-NP in a simple aquatic food web.

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Chapter 5
Annexes

Annex 1

Table 4: Results of $EC_{20,72h}$ calculation for Raphidocelis subcapitata and Chlorella vulgaris with respective 95% confidence limits (CL).

	Raphidocelis subcapitata	*Chlorella vulgaris*
F0	39 µg/L (95% CL: 30-48 µg/L)	79 µg/L (95% CL: 52-90 µg/L)
F1	25 µg/L (95% CL: 16-34 µg/L)	>90 µg/L
F2	14 µg/L (95% CL: 7-20 µg/L)*	>90 µg/L
F3	>53 µg/L	>90 µg/L
F4	17 µg/L (95% CL:11-22 µg/L)**	>90 µg/L

For F2 and F4 of *Raphidocelis subcapitata*, an $EC_{50,72h}$ was also calculated:
*F2: $EC_{50,72h}$ = 27 µg/L (95% CL: 21-34 µg/L)
**F4: $EC_{50,72h}$ = 50 µg/L (95% CL: 42-59 µg/L)