Transformations of Silver Nanoparticles in Wastewater Effluents: Links to Ag Bioavailability

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ABSTRACT

As a result of the burgeoning nanotechnology industry, the number of uses of engineered silver nanoparticles (Ag NPs) in consumer products has risen significantly in recent years. Despite their utility as anti-bacterial agents, the 'nano-scale' properties of these materials may lead to eco-toxicological problems when they end up in the environment. Wastewater effluents represent one of the main routes through which Ag NPs can reach an aquatic environment, where they may potentially interact with aquatic life. However, in wastewaters, Ag NPs may undergo different chemical and physical transformations, which may alter the potential toxicity of these NPs towards aquatic organisms. The main objectives of this study are to characterize the Ag NPs in wastewater effluents and then to assess their interactions with a model organism, the green alga *Chlamydomonas reinhardtii*.

Experiments were conducted to distinguish the effect(s) of the wastewater matrix on the dissolution of Ag NPs and to determine whether the transformed NPs or the dissolved Ag species would be most bioavailable to *C. reinhardtii*. It was shown that in environmental matrices such as wastewater, the bioavailability of Ag^+ could be significantly or completely reduced—a conclusion that is in contrast with experiments performed with Ag^+ ions in simple biological media. The substantial reduction in the bioavailability of Ag^+ in wastewaters could be explained by the presence of high concentrations of organic/inorganic ligands, which would bind to Ag^+ and render it non-bioavailable. Another plausible explanation is the presence of various types of ions in wastewaters that would compete with Ag for biouptake. Nonetheless, Ag NPs did appear to be bioavailable to *C. reinhardtii* cells at higher concentrations, as observed from bioaccumulation experiments comparing dissolved Ag and Ag NPs. Since the biouptake of Ag

NPs could not be explained by free Ag^+ alone, it was speculated that complexed Ag^+ and/or small NPs could have contributed to biouptake. Overall, the results suggested that the water chemistry of wastewaters had a significant effect on the biouptake of ionic or particulate Ag, by changing their physicochemical states.

RÉSUMÉ

Durant ces dernières années, l'utilisation des nanoparticules manufacturées d'argent (Ag NPs) a augmenté considérablement suite au plein essor qu'a connu l'industrie de la nanotechnologie. Malgré leur grande utilité comme agents antibactériens, les propriétés de ces nanomatériaux peuvent conduire à de sérieux problèmes éco-toxicologique s'ils se retrouvent dans l'environnement. Les eaux usées constituent l'une des principales voies que les Ag NPs peuvent emprunter pour atteindre l'environnement aquatique et interagir avec les différents (mico)organismes qui y vivent. Cependant, dans les eaux usées les Ag NPs peuvent subir diverses transformations physicochimiques qui peuvent modifier leur toxicité potentielle vis-à-vis des organismes aquatiques.

Les objectifs principaux de cette étude étaient de caractériser les Ag NPs dans des effluents d'eaux usées et ensuite évaluer leurs interactions avec une algue verte *Chlamydomonas reinhardtii* considérée comme un organisme modèle.

Nous avons étudié l'effet de la matrice des eaux usées sur la dissolution des Ag NPs et la biodisponibilité des différentes formes (particulaire et dissoute) des Ag NPs pour *C. reinhardtii.* Dans les matrices environnementales telles que les eaux usées la biodisponibilité de Ag^+ serait réduite de façon significative, si ce n'est complète; une conclusion qui va à l'encontre les résultats des études similaires faites dans des matrices biologiques simples. Cette réduction notable de biodisponibilité de Ag^+ dans les eaux usées peut être expliquée par la présence de concentrations élevées de ligands organiques et inorganiques qui peuvent se lier fortement aux ions Ag^+ et inhiber ainsi l'interaction de ces derniers avec les organismes aquatiques. Une autre explication plausible serait la présence d'autres types d'ions qui entreraient en compétition avec Ag^+ pour la bioaccumulation par les (micro)organismes. Toutefois, à des concentrations élevées,

les expériences de bioaccumulation comparative de Ag NPs et Ag dissous ont montré une certaine biodisponibilité des Ag NPs pour les cellules de *C. reinhardtii*. La bioaccumulation des Ag NPs ne pouvant être expliquée par Ag^+ seul, on peut admettre que les complexes de Ag et/ou des nanoparticules de petites tailles peuvent contribuer à la bioaccumulation. En général, les résultats ont montré que la chimie des eaux usées a un effet significatif sur la bioaccumulation de l'argent ionique ou particulaire en modifiant leurs états physicochimiques.

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PREFACE AND CONTRIBUTION OF AUTHORS

This thesis was prepared in a manuscript-based format in accordance with the thesis preparation guidelines of McGill University. Chapter 1 presents an introduction to the thesis with a broad literature review and sets the objectives for the research. Chapter 2 contains the manuscript to be submitted to *Environmental Science: Nano* with the title of "Transformations of Silver Nanoparticles in the Wastewater Effluents: Links to Ag Bioavailability". The authors are Agil Azimzada and Nathalie Tufenkji from McGill University and Kevin J. Wilkinson from the University of Montreal. Chapter 3 states the research conclusions, discusses the challenges and provides a future outlook.

The experimental work, data analysis and manuscript drafting were performed by Agil Azimzada. Kevin Wilkinson and Nathalie Tufenkji provided continuous research supervision, technical advice and constructive feedback throughout the project. They also provided guidance and support in the preparation of the manuscript.

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CHAPTER 1: INTRODUCTION

1.1. Why do silver nanoparticles matter for the environment?

The extensive applications of nanotechnologies and growing market demand for nano-enabled products, have led to a significant growth in the production of engineered nanomaterials (ENMs) and their use in consumer products [1-3]. Among these ENMs, silver nanoparticles (Ag NPs) are one of the most commonly used nanoparticles, finding increasing applications in a wide range of market sectors ranging from textiles to medical devices [4-6]. Silver as an anti-bacterial agent has a long historical record of use, but the production of engineered Ag NPs has enabled the use of these materials in diverse applications [7]; the most notable areas of application are cosmetic products, textiles, food packaging, and health supplements [8, 9]. As of March 2015, silver nanoparticles were reported to be the most frequently used nanomaterials, as they have been found in at least 435 of a total of 1814 listed nano-enabled products, according to the Nanotechnology Consumer Products Inventory (CPI) [10]. Increasing penetration of ENMs—including Ag NPs—into the global marketplace will inevitably lead to their increased emission into the environment, during the manufacture, use and disposal phases of an ENM-enabled-product's life-cycle [11].

In spite of the increasing applications of Ag NPs, their potential implications for the environment and human health are not yet well understood [5, 12]. Uncertainties about the 'nano-specific' novel properties of these nanoparticles and their potentially hazardous biological impact have generated considerable safety concerns that will need to be addressed. Although there are a number of studies that have already shown the potential toxicity of Ag NPs on various living organisms, several challenges remain, including better understanding whether their

potential negative impact originates from the 'nano' aspect (i.e., size) of the nanoparticles or from their innate chemical characteristics (i.e., the fact that they are silver) [4, 13]. Although the negative implications of silver ion and colloidal silver are not new, as they have been discussed since the beginning of the 20th century [14], some studies suggest that the toxic effects from Ag NPs are derived from a release of Ag^{+} ions, without the direct involvement of the NPs in the toxic action [15]. However, there are also suggestions that the Ag NPs might be bioavailable for microorganisms as well as humans, and that they may be able to cause direct toxicity to cells [16].

Despite an abundance of nanotoxicology research in recent years, there is still a lack of systematic studies that are aimed at understanding the fate and transformations of ENMs in environmental media, along with investigations on how the transformed ENMs would impact living organisms. Ag NPs have been shown to undergo physical and chemical transformations when they end up in the environment [17]. Depending on which environmental compartment (e.g., soil, wastewater, and surface water) they end up in, the type and extent of the physicochemical transformation will be different. These differences in the physicochemical state of the NPs will in turn affect their bioavailability and associated potential toxicity. In addition, the fate and behavior of the ENMs are significantly influenced by their transport pathways and the site of interaction with the living organisms. Understanding the behavior of ENMs in the environment and their subsequent interaction with organisms is thus key to proper risk assessment and safe nanomaterial design. Considering the current lack of systematic studies in this field, a main goal of this thesis is to combine a comprehensive Ag NP characterization with bioavailability experiments that seek to explain the 'nano-scale' effects—if there are any—in an environmental compartment of great priority, i.e. wastewaters.

1.2. Wastewater effluents as an exposure pathway for silver nanoparticles

The potential origins of ENMs and their potential ecological effects are summarized in Figure 1 [18]. Typically, ENMs released from household and industrial commodities find their way through waste disposal streams and into wastewater treatment plants (WWTPs) [8, 19]. WWTPs represent a major source of ENMs for the environment, as the two major outlets from the treatment facilities—namely, wastewater effluent (liquid) and wastewater sludge (solid)—are discharged back into nature [20]. Although a major fraction of the ENMs entering WWTPs are removed during the primary and secondary treatment processes via the wastewater sludge, a small portion (5-15%) still may end up in surface waters, such as lakes and rivers, through the discharge of liquid effluent [20]. The possibility of increased nanomaterial exposure due to the discharge of wastewater sludge also cannot be disregarded, since the resulting biosolids containing ENMs can end up in landfills and incineration plants and may thus pose an ecological threat [8, 21]. Indeed, a recent study has shown that the incineration process can convert the Ag NPs that have been sulfidized in wastewater systems back to their original state, which may increase their environmental risk [22]. The implications related to the discharge of liquid effluents into surface waters can be direct and severe for aquatic organisms.

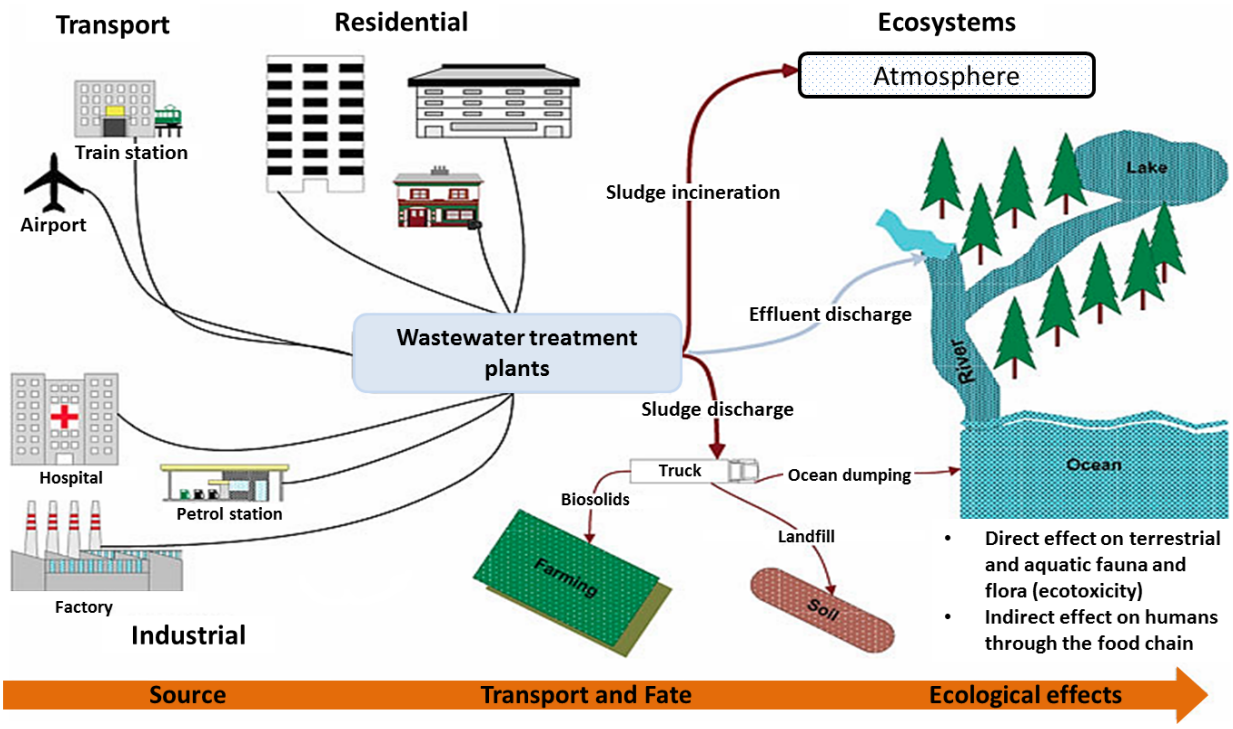

Figure 1. Sources, distribution and ecological effects of nanomaterials in wastewater systems [18]

Challenges still remain in the detection and quantification of ENMs in various environmental compartments; it is a particularly challenging task to assess environmentally relevant concentrations of ENMs in complex matrices, such as wastewaters or surface waters [23]. Because analytical data on ENM concentrations are scarce, predictive models are frequently used to estimate ENM concentrations [5, 12]. Based upon both analytical and modelling methodologies, concentrations of ENMs in the environment are very diverse. For example, whereas analytically measured concentrations of Ag NPs in wastewater effluents have ranged from 13 ng L^{-1} to 100 ng L^{-1}, estimations obtained from predictive models range from 16.4 ng L^{-1} to 65 µg L^{-1} [5, 24-26]. Similarly, estimations based upon predictive modelling

ranged from 2.8 ng L^{-1} to 619 ng L^{-1} for Ag NP concentrations in surface waters [27]. The variability in these estimations seems justifiable, given the diversity of factors affecting the release of ENMs from consumer or industrial products at various locations around the world. With the ever-growing penetration of nanotechnologies into the consumer marketplace, a future important increase in the emission of ENMs into the environment seems certain [10].

1.3. Physicochemical properties and behavior of silver nanoparticles

The sizes, shapes, surface chemistry and compositions of ENMs can be systematically tailored to achieve the required physicochemical properties for certain applications [28]. Although the physicochemical properties of ENMs provide them with higher functionality and efficiency compared to their bulk alternatives, there is growing number of studies showing that these same physicochemical properties can lead to adverse environmental implications, when ENMs are released into the environment [8]. Particle size is perhaps the most important feature of the nanoparticles, because at the nano-scale, the particles have an enhanced surface area, reactivity and transport capabilities as well as other associated 'nano-scale' features [29, 30]. Depending on the colloidal stability of the nanoparticles under environmental conditions—which will be mainly controlled by particle surface charge and morphology as well as several factors in the bulk media (e.g. *I*, pH, presence of ligands, temperature)—the particles may be subject to agglomeration/aggregation, dispersion or dissolution, which will change the particle size [17]. There are a variety of coatings used for Ag NPs to stabilize them against agglomeration, including citrate, carbonate, polyvinylpyrrolidine, polyacrylate and polyvinylamide [17, 31]. The coatings generally confer stability by building up an electrical charge on the NP surface or by steric stabilization.

Dissolution is another important process that is governed by the physicochemical properties of NPs. The dissolution mechanism involves the oxidation of metallic silver and the release of Ag^+ ions [17, 32]. The extent of NP dissolution depends on dissolved oxygen concentration, pH and presence of inorganic/organic ligands. Furthermore, a fraction of released Ag^+ ions may be precipitated in the presence of ligands, such as Cl^- and S^{2-}, again facilitating the conversion of the Ag NPs [33]. Moreover, the presence of natural organic matter in the medium can enhance the NP dissolution by complexing the released Ag^+ ions [16, 34]. All these processes can be summarized with the following chemical equations, where X and L denote a precipitating agent and a complexing organic/inorganic ligand, respectively.

$$2Ag_{(s)} + 1/2\ O_{2(aq)} + 2H^+_{(aq)} \leftrightarrow 2Ag^+_{(aq)} + H_2O_{(l)}$$

$$Ag^+_{(aq)} + X^{n-} \leftrightarrow Ag_nX_{(s)}$$

$$Ag^+_{(aq)} + L^{x-}_{(aq)} \leftrightarrow AgL^{(x-1)-}_{(aq)}$$

Since the release of Ag^+ is a major mechanism through which Ag NPs may induce their toxicity, the characterization of Ag NP dissolution should be a major part of any nano-toxicological research with Ag NPs.

1.4. Mechanistic considerations of the potential toxic action

Before discussing the processes that can participate in the transformation of Ag NPs in a wastewater, it is essential to consider the potential mechanisms leading to the toxic action of Ag NPs towards cells. A mechanistic understanding of NP-cell interaction can be a strong basis for evaluating NP bioavailability and its potential toxicity [28]. The bioavailability of a NP will be determined, in part, by its physicochemical properties—which will influence its physical or chemical speciation under a given set of environmental conditions. In addition, the physiological

state of the cell will influence the dynamics of interaction with either the NP or the species originating from it. Once the NP or related transformed species interact with a physiologically active site on the cell surface, they may affect the cell metabolism and disrupt the cellular homeostasis [35]. If the interaction is strong enough to overwhelm the stress responses, which arise due to a disruption in homeostasis, a toxic outcome will ensue [36]. The bioavailability of a NP can be inferred by characterizing a biological end-point; these biological end-points will be different for different organisms (e.g., algae, bacteria, and fish) and may include metal bioaccumulation (sorption or uptake), photosynthetic activity, motility, respiration and growth [35]. The assessment of metal bioaccumulation upon the exposure of NPs/metals is an insightful method to elucidate the mechanism of interaction between the NP and organism. This method is particularly useful because it can help answer the fiercely debated question of whether NP toxicity is caused by the direct interaction of the NPs or rather by providing a buffer for Ag^+ release from the NPs.

Previous studies have already shown the negative impact of Ag NPs in the environment and particularly, their role towards aquatic organisms such as bacteria, algae, fungi and fish [15, 37, 38]. Studies evaluating mechanistic scenarios for the interaction and toxic action of Ag NPs have been of particular interest. For example, as mentioned above, an open question remains as to whether the Ag NPs can have specific 'nano-effects' originating from their 'nano-properties' that could lead to toxic effects different from what could be predicted based on an 'only Ag^+' scenario. Although there is overwhelming evidence showing that Ag NP toxicity is mediated by Ag^+ release from its surface, there is no consensus on whether or not Ag NPs are a direct cause of enhanced toxicity [17]. For example, Navarro et al. [15] showed that Ag NP toxicity to *C. reinhardtii* resulted mainly from the release of dissolved Ag^+ ions. The study also suggested that

Ag NP toxicity could be enhanced if the NP interacted with the algal cell surface by facilitating the release of further Ag^+ ions. Piccapietra et al. [39] also attributed the main toxicity of Ag NPs to the dissolved Ag^+, suggesting that a limited number of Ag NPs were bioavailable to *C. reinhardtii* by either internalization or sorption to the cell surface. Similarly, although Leclerc and Wilkinson [40] observed nanoscale deposits of Ag inside the cell as well as on the cell surface, they suggested that these deposits were generated following the *in situ* reduction or precipitation of Ag^+. By measuring the induction of an Ag^+ (Cu^+) transporter, they were able to show that Ag bioaccumulation was largely due to the ionic forms of the metal. On the contrary, Miao et al. [41] suggested that Ag NP internalization into the *Ochromonas danica* cells was an important mechanism through which algal growth was significantly reduced. They showed that Ag NP uptake was very important even in the presence of Ag^+ binding ligands, such as cysteine, and concluded that Ag NPs inside the cells could contribute to toxicity. Finally, Fabrega et al. [42] showed that the toxicity of Ag NPs towards *Pseudomonas fluorescens* was caused by the intrinsic properties of the NPs rather than their dissolution. The toxic effect of the NPs was hypothesized to be caused by either uptake or cell membrane pitting and disruption.

As discussed, there are different conclusions from various studies examining the mechanistic action of the Ag NPs to cells. Some of these differences emerge because of objective causes in different studies, such as the type of organism or NP used. However, they may also have emerged due to a lack of control for the unpredictable loss of Ag to sources other than the biological cells. Such a lack of control could easily lead to different interpretations of the mechanisms leading to the uptake of the NPs. Another pitfall of these studies is that many are conducted using simple biological media, which cannot be considered as being 'environmentally relevant'. In reality, the transformation of Ag NPs in a biochemically rich environmental

medium, such as wastewater, and the biological effects occurring in such a medium will change the total dynamics of NP-cell interaction, by altering the bioavailability of Ag NPs and/or released Ag^+ ions. Consequently, in a wastewater medium, discussion of the NP-cell interaction mechanism needs to be expanded in order to account for the potential interaction of other transformed Ag species, which may include different particulate forms with various sizes (e.g., AgCl NPs, Ag_2S NPs) or dissolved forms (e.g., $AgCl_x^{1-x}$, $AgS_2O_3^-$) [16]. Therefore, it is imperative that Ag NPs are thoroughly characterized before the interaction with algae cells in the relevant medium.

1.5. Transformation of silver nanoparticles in wastewaters

Modelling studies have shown that the majority of Ag NPs incorporated into consumer products are released into wastewater systems and likely end up in WWTPs [5]. As these ENMs eventually end up in natural water bodies, they are likely to interact with the aquatic surfaces and biological species. Furthermore, there is evidence that the ENMs will persist in the environment and be subject to different environmental conditions, which may transform their physicochemical states [20]. Therefore, Ag NP speciation determinations in wastewaters are of key importance in understanding their bioavailability towards freshwater aquatic organisms.

Upon their release into the wastewater systems, Ag NPs will be subject to different environmental forces and may be transformed into various chemical species, such as dissolved Ag species, Ag_2O, Ag_2S and AgCl [8, 19, 20]. One of the most important processes that govern Ag NP transformations is sulfidation. Under anaerobic conditions, which are common in wastewater systems, the presence of high hydrogen sulfide concentrations are expected; the sulfides likely react with oxidized Ag to form more thermodynamically favourable Ag_2S species [19]. Indeed, previous studies have shown that Ag NPs are converted to Ag_2S NPs during their

transport through WWTPs and that the sulfidation process may start even earlier—for example, in urban sewer systems prior to their arrival at WWTPs [19]. It has been suggested that the Ag NP sulfidation process may follow either a heterogeneous mechanism (above 0.8 μM of sulfide) or a dissolution/precipitation mechanism (below 0.8 μM of sulfide), depending on the available sulfide concentrations [43]. The extent of NP sulfidation may differ depending on the NP size and coating, as well as the presence of available reactive sulfide species (H_2S, HS^-) [44]. There are studies suggesting that the sulfidation of Ag NPs passivates the outmost layer of NP surface and renders them more resistant to dissolution (release of free Ag^+ and other dissolved forms), thereby decreasing their potential toxicity. For example, the reduced toxicity associated with Ag NP sulfidation has been observed in experiments conducted on *Escherichia coli* [45]. In contrast, a different study showed the non-uniform nature of sulfidation reaction; i.e., sulfidation may occur only on the specific portions of NP surface, which can leave open the possibility for further dissolution [44].

The release of Ag NPs from consumer products may also lead to the formation of AgCl particles, via the leaching and subsequent scavenging of Ag^+ ions by chloride present in the medium. For instance, the formation of AgCl after the exposure of Ag NP containing socks to a hypochlorite/detergent solution has been demonstrated, which implies that AgCl can be formed before entering the wastewater systems. In this case, the conversion to insoluble AgCl likely limits the antibacterial activity and potential toxicity of the NPs [46]. In wastewaters, however, the presence of high chloride concentrations may modify the speciation from insoluble AgCl particles to soluble $AgCl_x^{1-x}$ complexes, which have been shown to be potentially bioavailable [35, 47]. Furthermore, the abundance of natural organic matter and the conditions conducive to Ag^0 oxidation—as determined by pH, dissolved oxygen concentration and lighting—can pave

the way for the formation of dissolved silver species, including complexes and free Ag^+ ions. Although free Ag^+ ions have been shown to be readily bioavailable, the complexation of Ag^+ ions with organic or inorganic ligands may significantly limit—if not completely reduce—their bioavailability [17, 48]. In a biochemically rich medium, such as a wastewater, Ag ligands may include S-containing compounds, biological macromolecules (DNA, proteins), and inorganic species, such as CO_3^{2-}, F^-, $S_2O_3^{2-}$, SO_4^{2-}, and PO_4^{3-}. Despite the general consensus—which is mostly derived from Biotic Ligand Model (BLM)—that free Ag^+ ions are the main Ag species that is bioavailable, there are a number of studies showing the bioavailability of Ag complexes [35], which retain a capacity for interacting with biological targets and being internalized by the cells [36].

1.6. *Chlamydomonas reinhardtii* as a model organism in surface waters

The green alga, *Chlamydomonas reinhardtii,* is one of the widely used model organisms in research. It is a unicellular organism that can be found in freshwaters and soils. The use of *C. reinhardtii* offers several advantages: it is easy to culture, it has a simple life-cycle, and there are a vast array of techniques for its study by molecular techniques [49]. The fact that *C. reinhardtii* are present in surface waters—where they may potentially interact with Ag NPs coming along from wastewater effluents—makes the use of this type of alga environmentally relevant in our study. With the exposure of green algae to Ag NPs, different biological or toxicological end-points, which may include viability, growth, photosynthetic activity, bioaccumulation and gene expression, can be investigated [15, 35, 39, 40, 42].

1.7. Algae related considerations in ENM-cell interaction

Algal cells are surrounded by cell walls, which are an important barrier to the physical penetration of ENMs into the cells [36]. Indeed, the importance of the cell wall in protecting the

algal cells from Ag NP internalization has been demonstrated by Piccapietra et al. [39], as they have found higher rate of Ag internalization in the cell-wall-free strain of *C. reinhardtii* as compared to the wild-type strain. Nonetheless, it was also shown that cell walls are semi-permeable and that they may still allow the passage of entities on the nano-scale [15]. Finally, it has been shown that the emergence of a toxic outcome does not necessarily require the internalization of NPs into the cells [36].

The subsequent encounter of ENMs with cells occurs at the plasma membrane (or cell membrane) interface, which defines the boundaries of a cell [28]. This bio-physical interaction may include processes that disturb the phospholipid bilayers [36]. Depending on the ENM surface characteristics, this interaction may potentially lead to gelation or "hole" formation in the phospholipid bilayer. Furthermore, ENMs could also induce physical responses in the membrane protein complexes, such as ion channels, which can be physically blocked by nanomaterials. Note that ion channels in the plasma membrane may also provide a potential pathway for ionic metal species (perhaps even nanomaterials) to enter into the cell, where damage could be inflicted on different cellular organelles [28].

The molecular mechanisms enabling the biological uptake of Ag by cells has not been strongly examined, however, there is evidence that the interaction of Ag with a plasma membrane—regardless of whether the uptake occurs or not—will likely lead to the activation of a stress response in the cells [16]. If the ENM interaction is so intense that the stress response is overcome, then a toxic outcome will ensue [36]. Another important consideration that should be accounted for is how the interaction of algae or algal exudates (organic substances excreted from algae) with Ag NPs would influence their speciation [50, 51]. There are some studies showing the effects of natural organic matter on Ag NP bioavailability; however, it is essential that the

specific effects of algal exudates on Ag NP dissolution and speciation are investigated to gain a better understanding of Ag NP bioavailability in the interaction with algae.

1.8. Thesis objectives

The objectives of this thesis were (i) to develop comprehensive understanding of Ag NP dissolution and speciation and (ii) to investigate the bioavailability of Ag NPs to the green alga, *Chlamydomonas reinhardtii* in a wastewater effluent. The dissolution of Ag NPs was carefully characterized using single particle inductively coupled plasma mass spectrometry (SP-ICP-MS) in both wastewaters and other simple media. The dissolved Ag species were investigated for their potential complexation by coupling an ion-exchange technique (IET) to the ICP-MS. Additionally, the influence of algal exudates on the speciation of Ag NP was evaluated, based on the above techniques. The biouptake of silver from $AgNO_3$ and Ag NPs in wastewater and control media (algae growth media of modified salts) was quantitatively analyzed by using bioaccumulation experiments with *C. reinhardtii* in an attempt to understand the effect(s) of wastewater on Ag NP bioavailability as well as to observe any arising differences between the two forms of silver with regards to their biological uptake.

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CHAPTER 2: Transformations of Silver Nanoparticles in Wastewater Effluents: Links to Ag Bioavailability

1. Introduction

The extensive use of silver nanoparticles (Ag NPs) in consumer products and growing market demand has led to a significant growth in their production. Silver has a long historical record as an anti-bacterial agent [1-3], but the production of engineered Ag NPs has enabled the use of these materials in diverse market sectors, including textiles, cosmetics, food packaging and health supplements [4]. A major fraction of consumer products containing Ag NPs eventually find their way through waste disposal streams and into wastewater treatment plants [5-10]. Therefore, wastewater effluents represent a major route through which NPs can reach surface waters [5, 11], where aquatic organisms can be impacted.

Increasing emissions of Ag NPs have prompted discussions on the implications for the environment and human health [12-14]. A number of studies have been carried out to evaluate the bioavailability and toxicity of Ag NPs for various living organisms, but several challenges remain, including better understanding as to whether their impact is derived from the direct interaction of Ag NPs, the indirect release of Ag ions, or both [15]. For instance, Fabrega *et al.* [16] and Miao *et al.* [17] suggested that toxicity was directly attributed to the NPs, whereas Navarro *et al.* [18] and Piccapietra *et al.* [19] attributed the main toxicity of Ag NPs to the release of Ag^+. There are also suggestions that Ag^+ and other dissolved Ag species could be generated locally at the biological interface following its interaction with Ag NPs [15, 18]. Indeed, Leclerc and Wilkinson [20] showed that the bioaccumulation of Ag NPs was largely due

to their interfacial dissolution and that Ag deposits (nanoparticles) were generated following the *in situ* reduction and precipitation of the internalized Ag^+.

As discussed, there is a lack of consensus on the possible mechanisms through which Ag NPs become bioavailable and induce their toxic effects. The different conclusions may have arisen in part, due to differences in the type of Ag NPs, the organisms used in the different studies or the biological or toxicological endpoints tested. Furthermore, there is evidence that Ag NPs undergo near continual transformations in the environment through biotic or abiotic processes — the particular type and extent of the transformation typically being determined by the specific pathway and destination of the NP [5, 9, 10]. Any change in the physicochemical state of the NP will in turn likely impact its potential toxicity, as the bioavailability depends on chemical speciation (complexation and oxidation number), diffusive fluxes (related to NP size) and particle coating [21-23]. In a biochemically rich matrix of wastewater, Ag NPs may easily be transformed into numerous chemical species, including free Ag and Ag complexes, Ag_2O, Ag_2S and AgCl [24-27]. Indeed, the evidence suggests that sulfidation is a prevalent process in wastewaters where passivation of the outermost layer of an Ag NP will render the NP more resistant to dissolution [25, 28, 29]. In addition, the abundance of organic/inorganic ligands in wastewaters may reduce the bioavailability of the released/available Ag^+ through complexation [13, 26], although it has also been shown that some Ag^+ complexes can be bioavailable [30, 31].

Given the variety of environmental factors that can affect the transformations of Ag NPs in wastewaters, it is essential to rigorously characterize the different Ag forms prior to their interaction with a biological cell. It is also important to assess the bioavailability of NPs in complex, real media (i.e., wastewater or surface water), where the biological interaction can occur — as opposed to the simplified biological media in which the majority of experiments are

performed. The combination of a rigorous NP characterization with experiments evaluating biological availability is necessary to gain a comprehensive insight into the risk of Ag NPs in complex environmental media such as wastewaters. In such a case, the scope of the discussion on 'whether the NP or the ion will lead to a given observation' will be extended to fully take into account the complex nature of NP speciation in wastewaters.

Consequently, the objectives of this study were both to develop an in-depth understanding of Ag NP dissolution and speciation and to investigate the bioavailability of Ag NPs in an environmentally relevant medium of high importance, i.e. a wastewater effluent. Based upon the understanding gained from the characterization and the bioavailability experiments, we hope to gain further insight into the nano-specific interactions of Ag NPs.

2. Materials and Methods

Materials. All chemical reagents were purchased from Sigma-Aldrich (ACS reagent grade), except for K_2HPO_4 and KH_2PO_4 (ACS reagent grade; Fisher Chemical), HCl (trace metal grade; Fisher Chemical), HNO_3 (67-70 %; BDH Aristar Ultra), NaOH (Acros Organics), tris-(hydroxymethyl)-aminomethane (BDH USP/EP grade, VWR) and $AgNO_3$ (1000 mg Ag L^{-1}; ICP-MS standard; Inorganic Ventures). Polypropylene tubes (15 and 50 mL) from Fisher Scientific and polycarbonate shaker flasks (1 L) from VWR were used. Citrate/tannic acid coated Ag NPs (50 nm) were acquired from Nanocomposix (Econix silver) as an aqueous suspension with a nominal Ag concentration of 2.19 g L^{-1}.

Wastewater effluent samples were collected from a municipal wastewater treatment plant in Montreal, Quebec, Canada. Milli-Q water (resistivity >18.2 MΩ cm, organic carbon <2 μ g C

L^{-1}) was used for dilutions and media preparation. Further chemical characterization is provided in Supplementary Information (Table S1). A simple biological medium, modified-TAP (M-TAP) (based on [32]) was used as a control medium in the dissolution and bioaccumulation experiments. M-TAP was prepared by diluting the salt composition (4x) of the standard tris-acetate-phosphate (TAP) algal growth medium and by substituting Cl^- by SO_4^{2-} and NO_3^- in order to avoid precipitation of Ag^+ ions (see detailed protocol in Table S2).

To avoid metal contamination, flasks were soaked in 2 % v/v HNO_3 for 24 hours, before being well rinsed with Milli-Q water and dried under laminar flow for experimental use. To avoid biological contamination, materials and media used in the preparation of the algal growth media were autoclaved prior to use. Cysteine solutions were freshly prepared before use.

Nanoparticle Characterization. Ag NPs were characterized for their aggregation and dissolution using Inductively Coupled Plasma Mass Spectrometry (NexION 300X ICP-MS) in single particle mode (SP-ICP-MS). Nanoparticle characterizations were performed in Milli-Q water (MQW), modified-TAP (M-TAP) and the wastewater effluent (WW) media over short term (up to 75 min) and/or long term (up to 7 day) exposures. For a few experiments designed to assess the effect of algal exudates on the dissolution of the Ag NPs, M-TAP solutions containing algae (0.15 cm^2 mL^{-1}, ca. 65,000 cells mL^{-1}, 1 hour exposure) but no Ag, were filtered over 3.0 μm nitrocellulose filter membranes (SSWP, Millipore). The filtrates, which were assumed to contain algal exudates but no algae, were then used as the NP exposure medium. SP-ICP-MS measurements were conducted in triplicate with Ag NPs using Ag concentrations of 100 ng L^{-1} or 400 ng L^{-1}. For the transport efficiency measurements, a NIST (National Institute of Standards and Technology standards) reference (RM 8013) of 50 mg L^{-1} citrate stabilized gold NP with a nominal diameter of 60 nm was used. For the sample measurements, the dwell time was set to

500 µs with no settling time and the data were acquired over 50 s. Syngistix nano application module integrated into the NexION software (version 1.1.4624) was used to acquire time-resolved signals. To ensure the reliability of the measurements, blanks and quality control NPs (NIST 1640a) were run regularly.

As a complementary approach to estimate dissolution, ultracentrifugation experiments were performed using 25-200 µg L^{-1} of Ag NPs in wastewater effluent after 1 day of exposure. In that case, 6 mL suspensions were centrifuged at 280,000x*g* for 15 mins using a Sorvall mTX 150 Micro-Ultracentrifuge (ThermoFisher). Five mL of supernatant was carefully removed from each tube in order to avoid disturbing the pellets. NP dissolution was determined by subtracting Ag in the supernatant from total Ag in the initial samples.

Dissolved Ag speciation. An ion exchange (IE) resin (Chelex-100 resin, 50-100 mesh, wet capacity of 0.40 meq mL^{-1}, Sigma) was coupled to the ICP-MS [21, 33] in order to gain further insight into the forms of dissolved Ag in the different media. Dissolved Ag was analyzed by SP-ICP-MS before and after the on-line coupling with the IE resin. The assumption was that the IE resin removed free Ag^+ ions and/or weakly-bound Ag^+ complexes. Visual MINTEQ software was used for the determination of Ag^+ speciation in an M-TAP (control) medium (with known salt concentrations)

Algal Cultures. In this study, *Chlamydomonas reinhardtii* (wild type C125 from the *Chlamydomonas* resource center) was used as a model organism. Algal culturing [20, 32] involved two transfer steps. First, 75 mL of a defined algal growth medium (4× diluted tris-acetate-phosphate medium (TAP)) was inoculated with *C. reinhardtii* cells from a Petri dish (agar with TAP). The inoculated algal suspension was incubated at 20 °C under 24 h lighting

with 100 rpm of rotary agitation, until the algae reached their exponential growth phase [20]. At this time, an aliquot of the algal culture was transferred to a fresh 400 mL of algal growth medium (4× diluted TAP) and incubated until a new exponential growth phase was reached. Cell numbers were measured using a Multisizer 3 particle counter (Beckman Coulter). Measured cell densities (number or surface area) were used to determine the growth phase as well as to ensure constant algal cell densities in the experimental suspensions. To harvest algal cells for the experiments, four algal suspensions of 50 mL were centrifuged at 3,000x*g* for 3 min. The algal pellets were washed with M-TAP solution and the supernatant was discarded. This washing procedure was repeated twice (2 min of centrifugation each time) and the algal pellets were eventually combined in a single 10 mL of algal suspension.

Bioavailability experiments. Ag NPs (or $AgNO_3$) were stabilized in the media of interest—M-TAP or the wastewater effluent—for 24 hours prior to exposure of the algae. Exposure media were covered with aluminum foil to protect them from light and agitated at 100 rpm. Bioaccumulation experiments (technical triplicates) were initiated by adding a small number of algal cells from the concentrated algal stock solution into the exposure medium (containing Ag) in order to obtain a final cell density of 0.15 cm^2 mL^{-1} (ca. 65,000 cells mL^{-1}) [20]. In a previous study, this cell density was shown to be low enough to ensure that Ag concentrations did not decrease significantly over the duration of the short term experiments [20]. Cysteine was used to stop (or significantly reduce) bioaccumulation at a selected time-point, as it can easily bind to Ag^+ ion and limit its bioavailability [18, 20, 34]. Triplicate, 45 mL samples from the exposure medium were mixed with 5 mL of 5×10^{-2} M cysteine, gently shaken for 1 min, and then filtered over 3.0 μm nitrocellulose filter membranes (SSWP, Millipore) (for $AgNO_3$ experiments) or 3.0 μm polycarbonate filter membranes (TSTP, Isopore) (for Ag NP

experiments). Filtered algal cells (diameter of ca. 6 µm) were washed three times with 5 mL of 5×10^{-3} M cysteine in order to remove any Ag remaining on the algal surface (in order to leave only Ag that had crossed the biological membrane). In the case of Ag NPs, independent control runs (before adding algae) were conducted to verify adsorptive losses to the filter membranes (polycarbonate), whereas in the case of $AgNO_3$, two superimposed filter membranes (nitrocellulose) [20] were employed, where the bottom membrane acted as the adsorption control.

Filter membranes were digested with 300 µL of 67-70 % HNO_3 (Aristar Ultra) for 8 hours at 85 °C using a DigiPREP digestion system (SCP Science). Samples were diluted to 3 % v/v HNO_3, one day before the ICP-MS measurements. Similarly, samples from the filtrates and the exposure media, before and after algal addition, at each time-point, were acidified and kept at 85 °C for 8 hours, before being diluted to 3 % v/v HNO_3. Mass balances were performed and any replicate outside the 85-115 % range was discarded.

3. Results and Discussion

3.1. Nanoparticle sizes

For 400 ng L^{-1} of Ag NPs in a wastewater effluent, mean diameters of 51.6 ± 0.1 nm, 48.6 ± 0.1 nm, 51.8 ± 0.4 nm and 51.0 ± 0.3 nm were determined after 1, 3, 5 and 7 days, respectively. These SP-ICP-MS results showed that the particle diameters did not change significantly over time and were in good agreement with the original particle size of 54.7 ± 6.6 nm, provided by the manufacturer (determined by transmission electron microscopy). Additional measurements obtained in the wastewater effluent, in the M-TAP medium and in Milli-Q water

for NP concentrations of 100 and 400 ng L^{-1} are provided in Table S3. For measurements performed over 75 mins in the M-TAP medium (400 ng L^{-1} Ag NP), without and with algal exudates, the NPs had stable diameters of 53.6 ± 0.3 nm and 51.9 ± 1.0 nm, respectively.

3.2. Nanoparticle dissolution

Ag NP dissolution was determined by SP-ICP-MS in the wastewater, M-TAP and Milli-Q water media (Fig. 2). Total Ag concentrations in the experimental tubes decreased slightly (0-4% for 10 µg L^{-1} of Ag; up to 30 % for 100 ng L^{-1} of Ag; Fig. S1), likely due to adsorptive losses. When measured concentrations of dissolved Ag were normalized by the measured concentrations of total Ag, it was possible to conclude that particle dissolution increased with time (Fig. 3). Indeed, for 100 ng L^{-1} of the Ag NP in wastewater, dissolution increased dramatically, with the proportion of dissolved Ag (includes free and complexed Ag^{+}) reaching 63 % after 7 days. For the same concentration of Ag NPs in Milli-Q water, the particles were much more stable with only 4.7 ± 2.9 % of dissolved Ag after 7 days, whereas in the algal growth media (M-TAP), the dissolution attained 27%, where it was stable for at least 3 days.

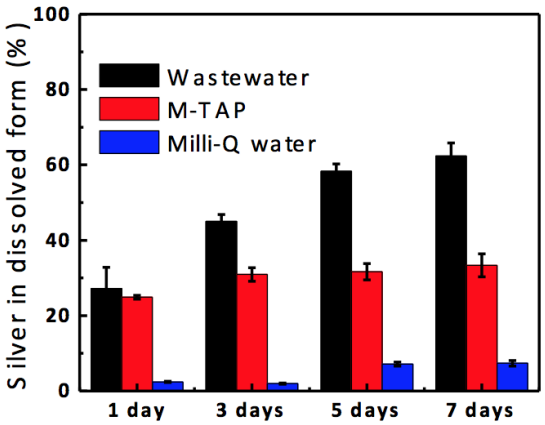

Figure 2. Dissolution of Ag NPs (initially 100 ng Ag L-1) in the wastewater effluent, modified-TAP media (M-TAP) and Milli-Q water media after 1, 3, 5 and 7 days as quantified by SP-ICP-

MS. Dissolved Ag is expressed as a proportion of the total measured Ag (mean ± standard deviation, n=3).

The effect of algal exudates on the dissolution of the Ag NPs is shown in Figure 3. For experiments occurring over several days, dissolution (%) nearly doubled in the pre-inoculated M-TAP medium (i.e. with algal exudates) as compared to the standard M-TAP (Fig. 3A). Similar measurements were conducted to determine Ag NP dissolution at shorter time scales (within a 75 min period); the results showed that the dissolution was much greater than the control medium containing no exudates. The rapid effect of algal exudates on Ag NP dissolution was nearly immediate (Fig. 3B).

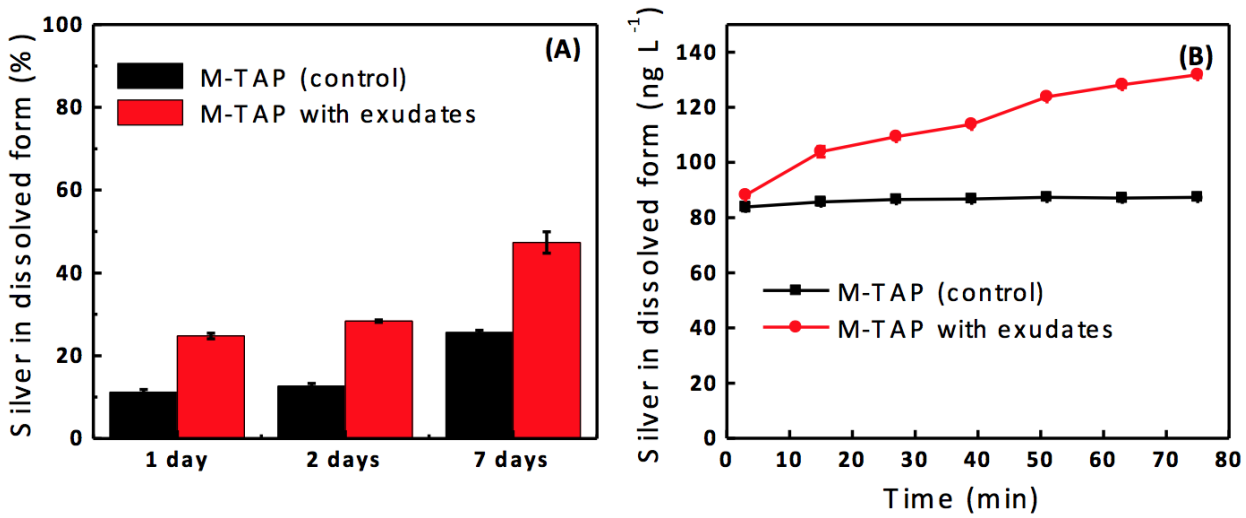

Figure 3. Dissolution of Ag NPs (nominally 400 ng L^{-1}) in the modified-TAP in the presence and absence of algal exudates. A) Proportion of Ag (%) in dissolved form (mean ± standard deviation, n=3) after 1, 2, or 7 days and B) Short term dissolution measurements (mean ± standard deviation, n=3) performed over 75 mins and measured in the M-TAP solution, with or without algal exudates. Determinations of dissolved Ag were made by SP-ICP-MS.

The observation that Ag NP dissolution increased dramatically in the biochemically rich matrices—either in the wastewater medium or in the algal growth medium containing algal exudates—can be explained by the complexation of free Ag^+ in solution [18, 28, 34-36], which will drive both the oxidation of the Ag NPs and the desorption of surface bound Ag^+ [33]. Although this explanation is entirely consistent with chemical thermodynamics [37, 38] and the observations of increasing dissolution, it is necessary to acknowledge that the quantification of dissolved Ag can also be influenced by the size detection limits of the SP-ICP-MS, which are approximately 16 nm for Ag NPs (for our instrument, under the conditions applied here). Ag NPs that are smaller than 16 nm will necessarily be characterized as "dissolved Ag", resulting in a potential overestimation of Ag NP dissolution. Furthermore, when using SP-ICP-MS, size detection limits will increase with increasing dissolved Ag [39], making particle solubilisation harder to detect for the most soluble NPs. Nonetheless, the use of Ag NPs that were significantly larger than the size detection limits and the observation that particle diameters were relatively stable over time are evidence supporting the validity of the SP-ICP-MS measurements.

To validate the SP-ICP-MS results, several complementary ultracentrifugation experiments were performed using 25, 50 and 200 μg L^{-1} of Ag NPs that were equilibrated for 1 day in the wastewater (Table 1). Following 15 mins of ultracentrifugation at 280,000x*g*, Ag concentrations in the supernatant were 1.7 ± 0.3 % of the initial concentrations, in contrast to the 27.2 ± 5.6 % that was determined by SP-ICP-MS, implying that very little dissolution occurred. Given that the techniques function at very different concentration levels (ppb vs. ppt), SP-ICP-MS is likely to provide an upper limit for the dissolution, whereas ultracentrifugation is more likely to give a lower limit. Indeed, although very little Ag was detected in the supernatant following ultracentrifugation, measured initial concentrations were significantly lower (ca. 50-

60%) than nominal concentrations. It is reasonable to assume that adsorptive losses to the container walls of the dissolved (positively charged) Ag occur to a greater extent than that of the highly stabilized (negatively charged) NPs, in which case, ultracentrifugation would underestimate dissolution. In addition, the high dilution requirement for the samples analysed by SP-ICP-MS is likely to contribute to the higher % dissolution observed by this technique, since Ag NP dissolution is well documented to increase with decreasing particle concentration [33, 40]. The SP-ICP-MS observation that NP sizes did not decrease significantly, even after 7 days in the wastewater, suggests that either dissolution did not occur homogeneously among the particles (some particles dissolved while others did not (see Fig. S2)) or that the particle dissolution was contributing to increased size detection limits that would effectively mask the smaller Ag NP in the complex media. In summary, the combined results indicate that (i) only a minority of the Ag NPs were soluble; (ii) particle dissolution increased at the lower particle concentrations and in the complex media; and (iii) particle dissolution increased with time.

Table 1. Dissolution of Ag NPs in a wastewater effluent medium after 1 day of exposure as characterized by ultracentrifugation experiments (280,000x*g*; 15 mins). Dissolved Ag is expressed as percentage of the total Ag determined before ultracentrifugation.

Total Ag *μg L^{-1}*	**Dissolution** *%*		
25	1.4	±	0.4
50	1.9	±	0.2
200	1.9	±	0.1

3.3. Characterization of dissolved Ag

The bioavailability of trace metals is most often predicted by the concentration of free ion, irrespective of the metal complexes [30, 41]. Therefore, a semi-quantitative approach, where a (cat)ion-exchange resin was coupled to the SP-ICP-MS (IET-SP-ICP-MS) [21], was employed in order to further discriminate among Ag species in the dissolved fraction (i.e., <16 nm). The underlying assumption was that strong Ag complexes, anionic complexes and small NPs would pass through the resin column without being involved in the ion-exchange, whereas free Ag^+ and labile Ag complexes would exchange with the counter-ions present on the resin. In the simplified growth medium (M-TAP), approximately 75% of dissolved Ag species were attributed to the free or weakly-bound Ag fraction, whereas in the wastewater or in the M-TAP amended with algal exudates, over 82 % of the Ag did not react with the ion exchange column (i.e. Ag was mainly found as either strong Ag complexes and/or small NPs) (Fig. 4). Given that free Ag and labile Ag complexes are far more likely to be bioavailable [15, 18, 30, 41] — and if the only mechanism of bioavailability for nanoparticulate Ag is dissolution — it is possible to predict that the Ag NPs will be far less bioavailable in the wastewater (or in the growth medium) and that bioavailable Ag will decrease with time.

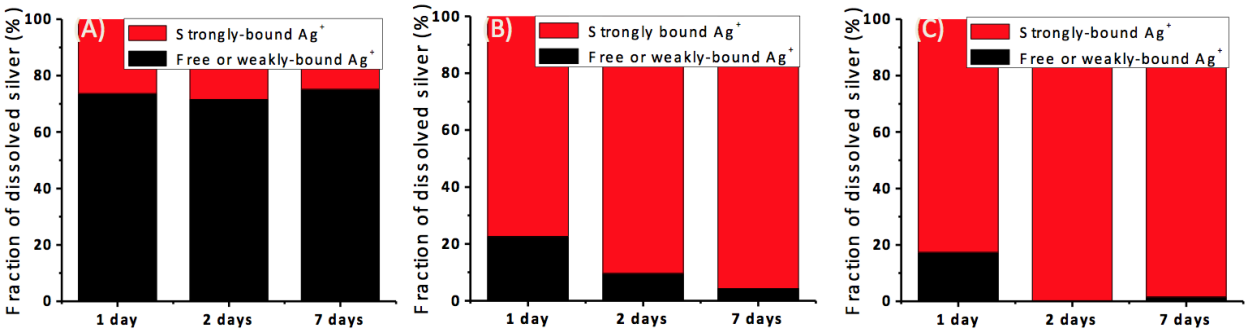

Figure 4. Ag speciation determinations for dissolved Ag species (from Ag NPs) based on IET-SP-ICP-MS in A) modified-TAP growth medium B) modified-TAP in the presence of algal exudates and C) wastewater effluent.

3.4. Ag bioavailability in the wastewater

Finally, metal uptake by the green algae *C. reinhardtii* was used as a basis to estimate the bioavailability of the different forms of Ag. Cells were exposed to $AgNO_3$ or Ag NPs in either the M-TAP or the wastewater effluent, separated by filtration (3.0 µm), then washed with 5×10^{-3} M cysteine to remove Ag bound to the outside of the cell. One of the challenges in bioaccumulation experiments is to quantitatively account for Ag losses to the filtration membrane, which may eventually lead to an overestimation of the biologically internalized Ag. Indeed, preliminary experiments showed that sorptive Ag losses could be significant (Fig. S3) and were highly dependent on the initial form of Ag in the exposure medium (ions or NPs). Since the use of nitrocellulose (depth) filters resulted in large losses of Ag NPs (up to 80% loss at the lower concentrations; Fig. S3), polycarbonate filters were employed for the bioaccumulation experiments, with independent control runs to estimate adsorptive losses. For exposures to ionic Ag, superimposed nitrocellulose filters were used, where Ag measured on the bottom filter could be clearly attributed to adsorptive losses. Nonetheless, in spite of these precautions, extrapolation of the Ag biouptake to the y-axis (0 min) still yielded non-zero values

for bioaccumulation in some cases. For this reason, and in agreement with common literature practice, results were analyzed using the metal uptake fluxes (slopes of the biouptake curves). Since metal uptake fluxes represent the rate of biouptake rather than its absolute measurement at a fixed timepoint, they can provide a more flexible and reliable basis for the comparison of bioavailabilities of different Ag forms in various media.

Algae were first exposed to 0.2-1.0 μmol L^{-1} of $AgNO_3$ in the M-TAP medium over 40 min. Biouptake increased linearly with time (Fig. S4), consistent with the Biotic Ligand Model (BLM) and previous literature results [20, 31]. When algae were exposed to similar concentrations (0.2-1.3 μmol L^{-1}) of $AgNO_3$ in the wastewater effluent (Fig. 5B), much lower Ag internalization fluxes were observed, with fluxes that were not significant at 0.2 and 0.5 μmol Ag L^{-1} but were small with a positive flux at 1.0 and 1.3 μmol Ag L^{-1}. The results clearly demonstrated that the bioavailability of Ag was largely or completely reduced when algae were exposed to $AgNO_3$ in a wastewater medium. The possible causes for reduced bioavailability in a wastewater medium include i) the **complexation** of Ag^+ ions by organic/inorganic ligands that render them non-bioavailable and ii) the presence of numerous ions in a wastewater medium that compete for Ag biouptake (**competition**) [42-44]. In contrast with the majority of previous studies where bioaccumulation experiments were conducted in simple growth media [18-20], Ag^+ bioavailability in the wastewater was greatly reduced due to the complex nature of the medium.

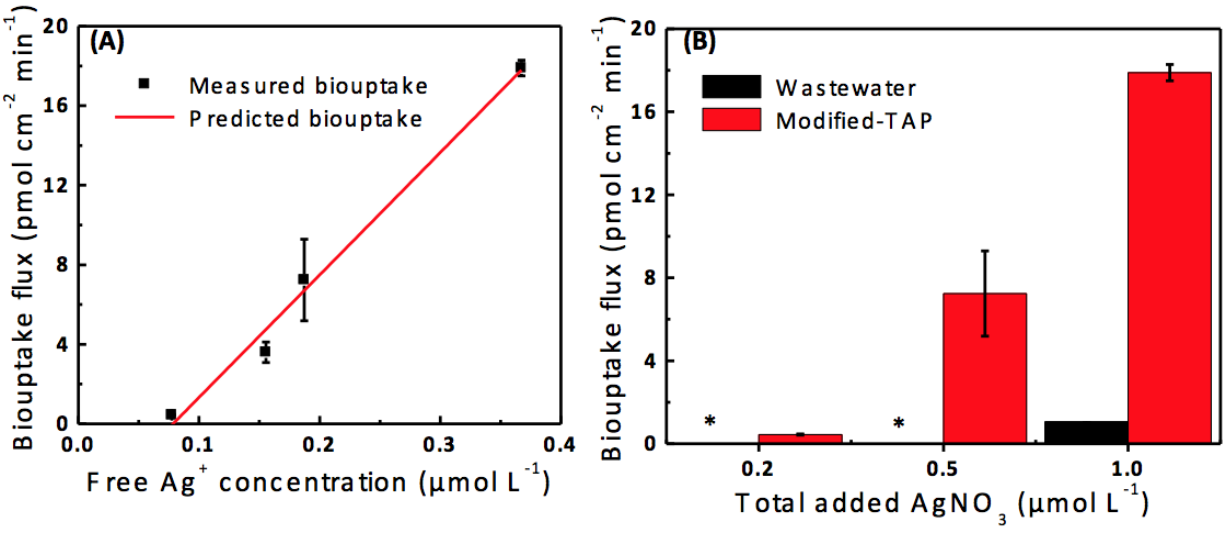

Figure 5. Determined biouptake fluxes (mean ± standard error, n=3) for algal cells (*C. reinhardtii*) exposed to $AgNO_3$ A) as a function of free Ag^+ concentrations (in modified-TAP predicted using Visual MINTEQ) and B) in modified-TAP and wastewater effluent media as a function of total added Ag. (*) refers to fluxes determined in the wastewater that were not significantly different ($p<0.05$) from zero.

Due to the low exposure concentrations, biouptake fluxes for the algae exposed to Ag NPs were more challenging to quantify. The use of polycarbonate filters for the Ag NP suspensions did minimize sorptive losses to less than 5 % (for [Ag] as low as 0.2 µmol L^{-1}) (Fig. S3). At the lowest exposure concentrations of Ag NPs (0.5 and 0.8 µmol L^{-1}), biouptake fluxes were not significantly different from 0 (one-tailed t-test; $p<0.05$) (Table S4). However, when biouptake experiments were performed using 1.3 µmol L^{-1} (or 2.0 µmol L^{-1}, see Table S4) of Ag NPs, uptake fluxes were significantly greater than those obtained following the addition of equivalent or lower concentrations of $AgNO_3$ (Fig. 6A&B). These results indicated that in the wastewater, the Ag from the NPs appeared to be bioavailable, potentially resulting from their

dissolution and production of small NPs or Ag complexes, as indicated by the SP-ICP-MS and ultracentrifugation experiments. Since the IET-ICP-MS experiments showed that most of the dissolved Ag resulting from the Ag NPs was in the form of strong complexes, biouptake in the wastewater could not be predicted from free Ag^+ alone, as was shown previously for this alga in simplified media [20]. Indeed, if SP-ICP-MS (Fig. 1) and IET-ICP-MS (Fig. 4C) experiments performed with Ag NPs were used as a basis to determine free Ag^+, concentrations would be below 0.08 μmol L^{-1} for 1.3 μmol L^{-1} of the Ag NP suspension. These are Ag^+ concentrations that are below the level of free Ag^+ shown to cause significant biouptake (with the protocol and instruments used in this study) (Fig. 5A). Therefore, the results suggest strongly that the production of smaller NPs or Ag complexes (resulting from transformations of the Ag NP in the wastewater) contributed to the overall biouptake.

It is important to note that Ag bioavailability did decrease substantially due to complexation (and/or competition) in the wastewater effluent in comparison to simple media (fluxes in M-TAP, Fig. S5, were greater than those determined in the wastewaters, Fig. 6B). Furthermore, little bioavailability could be directly attributed to either the ionic or particulate forms of Ag. We postulate that the Ag complexes and NPs function effectively as a Ag buffer with the capacity to locally buffer concentrations of free Ag^+. Nevertheless, as the results pointed out, the uptake of small Ag NP may occur, likely facilitated by physicochemical transformations of the NPs in the complex wastewater medium.

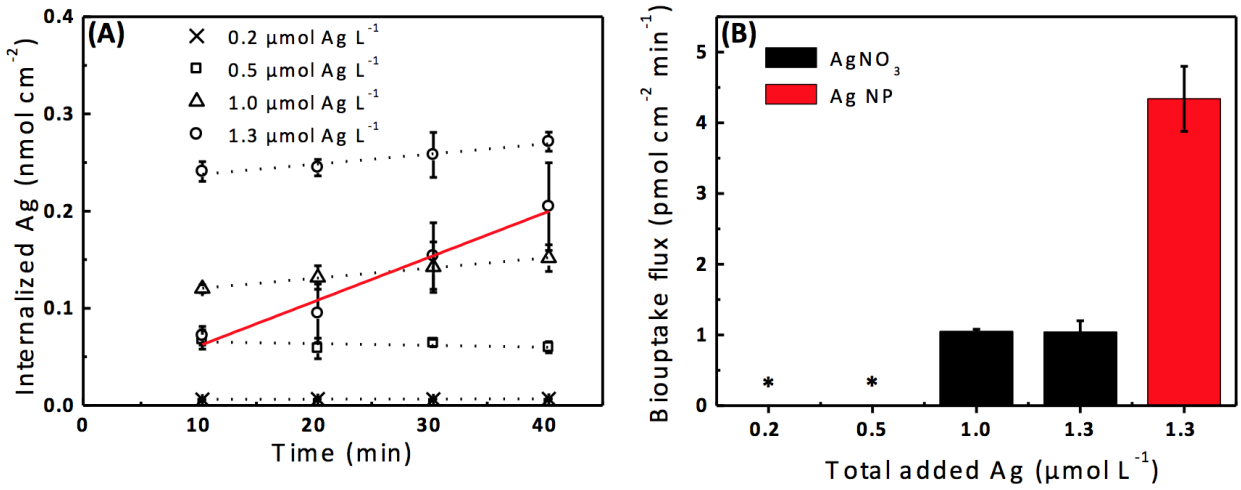

Figure 6. A) Measured Ag biouptake of algal cells (*C. reinhardtii*) (mean ± standard deviation, n=3) upon exposure to $AgNO_3$ (0.2, 0.5, 1.0 and 1.3 µmol L^{-1}) and Ag NPs (1.3 µmol L^{-1}) in wastewater effluents and B) corresponding biouptake fluxes (mean ± standard error, n=3) for each exposure. Solid (red) biouptake curve refers to Ag NP, while dotted curves refer to $AgNO_3$. Biouptake fluxes (B) correspond to the slopes determined from the internalization curves (i.e. Fig. 5A). (*) denotes fluxes determined for exposures to $AgNO_3$ that were not significantly different ($p<0.05$) from zero.

3.5. Environmental implications and challenges

In a wastewater effluent, Ag NPs appeared to be more bioavailable than could be predicted based solely upon the concentrations of free Ag. This observation is in stark contrast with previous studies [19, 20] that have been performed using simple biological media, where NP biouptake was largely attributed to dissolved Ag (presumably ionic Ag). Nonetheless, such a result is still consistent with previous studies [15, 18, 19] that have suggested that Ag NP bioavailability to algal cells could be mediated by the release of Ag^+ ions at the surface of the

organism (interfacial dissolution). Another implication of the study is that direct NP internalization might be possible in a wastewater medium, despite the reduction in the bioavailability of dissolved Ag species due to strong complexation and competition effects. Indeed, the potential increase in the concentration of small NPs over time in a wastewater medium—due to dissolution or dispersion of large NPs or reduction of dissolved Ag (and formation of small NPs)—may have been a potential cause of NP bioavailability that was higher than predicted based on free Ag concentrations. However, the analytical difficulties in distinguishing Ag complexes from very small Ag NP (i.e., those below the detection limits of the SP-ICP-MS) limits our capacity to draw more robust conclusions on the precise form of bioavailable Ag.

Despite the progress achieved in this study with respect to the reliability of the methodology and environmental relevance, several challenges still remain. One challenge is to develop a technique to detect, quantify and discriminate cell-internalized Ag (NPs or dissolved Ag) at the lowest ranges of environmentally relevant concentrations, as the biouptake quantification with the current methodology is limited below ca. 5-10 μg L^{-1}. NPs are subjected to different environmental factors that will affect their persistence in wastewaters. In a laboratory setting, simulation of real environmental conditions is a challenging task. Extraction of NP samples from an environmental matrix with minimal alterations to their physicochemical properties would be most useful for exposure experiments. Clearly, the numerous physicochemical transformations occurring in the complex wastewaters will greatly affect the bioavailability of the nanomaterials.

4. Acknowledgements

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2.5. Supplementary Information

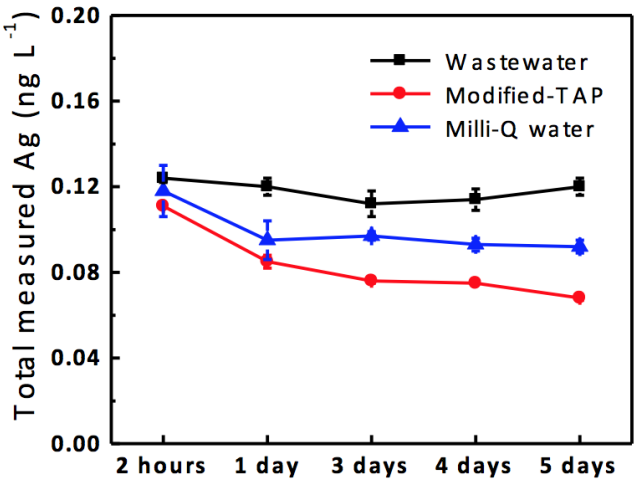

Figure S1. Total Ag measurements for a Ag NP suspension (starting concentration of 0.120 µg L^{-1}) after 2 hours, 1 day, 3 days, 4 days and 5 days of incubation in a polypropylene tube.

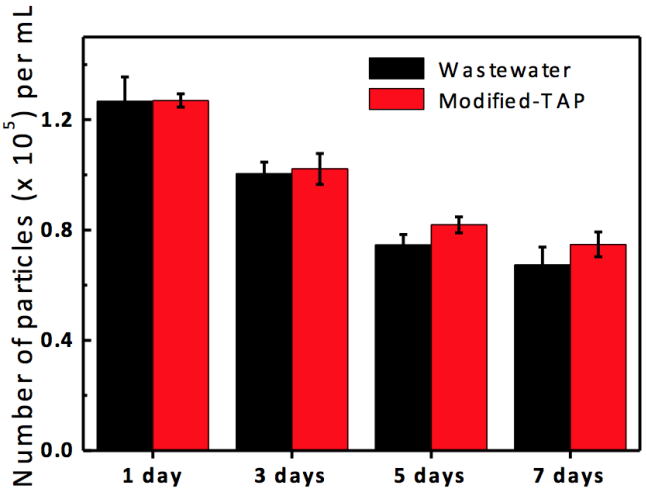

Figure S2. Particle number concentrations for a Ag NP suspension of 100 ng L^{-1} after 2 hours, 1 day, 3 days, 4 days and 5 days of incubation in wastewater and modified-TAP media.

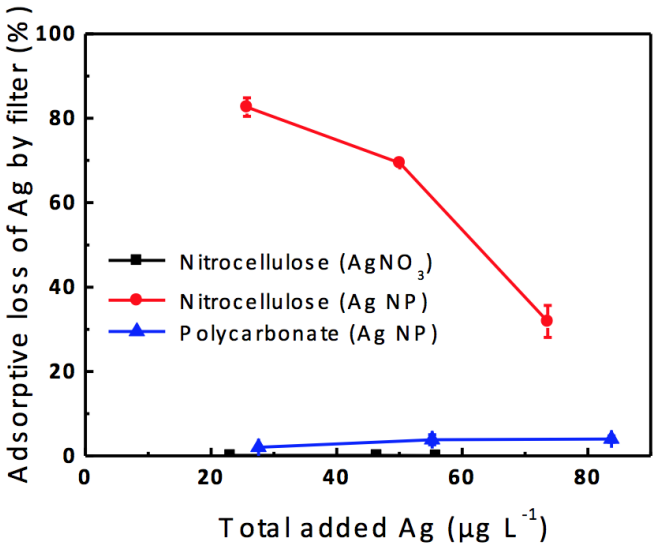

Figure S3. Total Ag retained by filter membranes (nitrocellulose or polycarbonate) of 3.0 µm pore size, after filtration of $AgNO_3$ solutions or Ag NP suspensions of various concentrations.

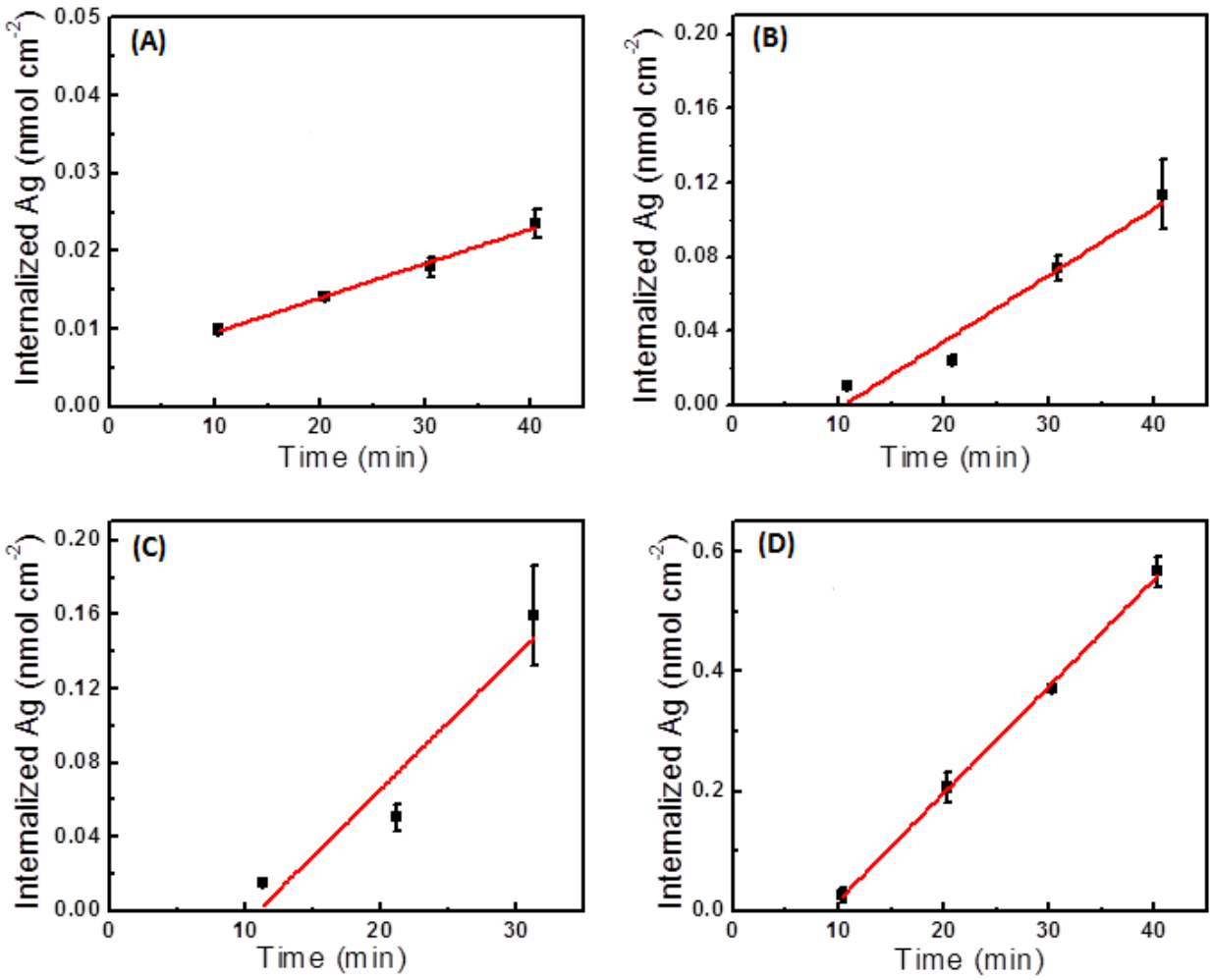

Figure S4. Measured Ag biouptake of algal cells (*C. reinhardtii*) upon exposure to $AgNO_3$ of A) 0.2 µmol L^{-1}, B) 0.5 µmol L^{-1}, C) 1.0 µmol L^{-1} and D) 1.3 µmol L^{-1} in modified-TAP (control) media as a function of time.

Table S1. Determinations of metal concentrations, pH and total organic carbon (TOC) in a filtered (3 µm, SSWP, Millipore) wastewater effluent sample collected at the wastewater treatment plant (WWTP) in Montreal, QC, Canada. Metal measurements were performed using semiquantitative analysis mode by ICP-MS. NM=not measured, ND= not detected

Metal	**Concentration** $\mu g\ L^{-1}$	**Metal**	**Concentration** $\mu g\ L^{-1}$	**pH**	**TOC** $mg\ L^{-1}$
Be	ND	Ag	0.002	7.7-7.8	16.2
B	40	Cd	ND		
Na	NM	Cs	0.014		
Mg	3327	Ba	2.06		
Al	1.0	La	0.001		
P	768	Ce	0.002		
K	4180	Pr	ND		
Ca	7656	Nd	0.004		
V	0.38	Sm	0.002		
Cr	ND	Eu	0.001		
Mn	2.1	Gd	0.017		
Fe	28.6	Dy	0.002		
Co	0.50	Ho	ND		
Ni	0.54	Er	0.001		
Cu	5.7	Tm	ND		
Zn	3.8	Yb	0.001		
Ga	0.007	Lu	ND		
As	0.26	Tl	0.020		
Se	0.83	Pb	0.12		
Rb	1.9	Th	0.030		
Sr	74.6	U	0.053		

Table S2. Protocol for the preparation of modified-TAP medium. MQW refers to Milli-Q water.

	Solution	Chemical/solution	Quantity/volume	Medium	pH
Step 1	Modified-Bei	$(NH_4)_2SO_4$	2.31 gr	500 mL MQW	-
		$Ca(NO_3)_2 * 4H_2O$	0.40 gr		
		$MgSO_4 * 7H_2O$	0.50 gr		
Step 2	Modified-Tris	Tris	29.04 gr	300 mL MQW	pH adjusted to 7
Step 3	Modified-TAP	Modified-Bei	20.0 mL	Complete to 800 mL with MQW	-
		Modified-Tris	5.0 mL		

Table S3. Mean particle sizes as measured by SP-ICP-MS for 400 ng L^{-1} and 100 ng L^{-1} of Ag NPs (starting concentrations) in a wastewater effluent, modified-TAP (M-TAP) and Milli-Q water media after 1, 3, 5 and 7 days of exposure.

	C_0 = 400 ng L^{-1}, Ag NP			**C_0 = 100 ng L^{-1}, Ag NP**		
Exposure time	**WW**	**M-TAP**	**Milli-Q water**	**WW**	**M-TAP**	**Milli-Q water**
day	*nm*	*nm*	*nm*	*nm*	*nm*	*nm*
1	51.6 ± 0.0	50.3 ± 3.7	50.5 ± 1.6	45.6 ± 0.2	40.1 ± 4.2	43.1 ± 0.6
3	48.6 ± 0.1	45.8 ± 5.5	48.5 ± 1.4	43.0 ± 0.7	37.0 ± 3.9	41.9 ± 0.3
5	51.8 ± 0.4	48.7 ± 3.2	48.8 ± 2.6	44.5 ± 0.3	39.9 ± 3.2	44.5 ± 0.7
7	51.0 ± 0.3	46.7 ± 3.3	48.6 ± 2.2	43.2 ± 0.5	37.9 ± 0.9	43.5 ± 0.5

Table S4. Biouptake fluxes for algal cells (*C. reinhardtii*) exposed to $AgNO_3$ and Ag NP in modified-TAP (control) and wastewater effluent media. *NS* refers to fluxes that were not significantly different ($p<0.05$) from zero.

Medium	**Ag source**	**Total Ag** *µmol L^{-1}*	**Biouptake flux** *pmol cm^{-2} min^{-1}*		
Modified-TAP	$AgNO_3$	0.2	0.44	±	0.03
Modified-TAP	$AgNO_3$	0.4	3.60	±	0.52
Modified-TAP	$AgNO_3$	0.5	7.24	±	2.05
Modified-TAP	$AgNO_3$	1.0	17.89	±	0.39
Wastewater effluent	$AgNO_3$	0.2		*NS*	
Wastewater effluent	$AgNO_3$	0.5		*NS*	
Wastewater effluent	$AgNO_3$	1.0	1.05	±	0.03
Wastewater effluent	$AgNO_3$	1.3	1.04	±	0.16
Wastewater effluent	Ag NP	0.5		*NS*	
Wastewater effluent	Ag NP	0.8		*NS*	
Wastewater effluent	Ag NP	1.3	4.34	±	0.46
Wastewater effluent	Ag NP	2.0	2.82	±	0.36

CHAPTER 3: CONCLUSIONS AND FUTURE OUTLOOK

The physicochemical behavior of Ag NPs is strongly influenced by their release pathways into the environment, as various biochemical constituents of the relevant medium will interact and modify the NPs. Wastewater effluents represent one of the major sources of NP release into freshwaters, where aquatic life can be impacted. Since NPs mainly arrive in freshwaters through wastewater systems, they will undergo significant physicochemical transformations during their transport that would eventually alter their bioavailability towards biological organisms.

Many of the implications of NP transformations in environmentally relevant media have previously been largely disregarded, as most of the bioavailability studies were conducted using simple biological media. Through characterization and bioavailability experiments, this study attempted to understand the bioavailability of Ag NPs in an environmentally important medium: namely, urban wastewater effluents. The results indeed demonstrated the strong impact of wastewater matrix on the transformation and bioavailability of Ag NPs. An important conclusion was that the bioavailability of dissolved Ag species (with $AgNO_3$ as a Ag source) was significantly or completely reduced in a wastewater medium—possibly due to the chemical speciation of Ag^+ that rendered them non-bioavailable; as well as the competition presented by the abundance of other dissolved metal species. In contrast, Ag NPs did appear to be bioavailable to *C. reinhardtii* cells in wastewater when the results from $AgNO_3$ and Ag NP bioexposure experiments were compared. Therefore, another implication of these results was that in a wastewater medium, the role of dissolution in the bioavailability of Ag NPs was limited, an observation in strong contrast with the studies performed using simple media.

The apparent bioavailability of Ag NPs could be enhanced with the effects of wastewater matrix. As the characterization measurements showed, Ag NPs in a wastewater medium were significantly more dissolved and/or possibly degraded (or dispersed) into smaller NPs (< 16 nm), compared to those in a simple biological medium (M-TAP). Although independent bioexposure experiments with $AgNO_3$ showed that dissolved Ag was largely non-bioavailable in wastewater, it is still conceivable that dissolved Ag generated from NPs—particularly, via the interaction with the algal cell interface—could be internalized. Another possibility is the direct internalization of NPs—a scenario facilitated with the likely increase in the concentration of NPs with smaller size range, upon exposure of original NPs to the wastewater matrix. Furthermore, it is possible that the mechanism of internalization for Ag NPs is different from that of dissolved Ag; this difference in turn may allow Ag NPs to be preferentially internalized and not be limited by the competition presented by the presence of other dissolved metal species (e.g., free ions).

This study demonstrated the importance of environmental factors in the transformation of Ag NPs and their bioavailability towards algae. It was concluded that the wastewater matrix can alter NPs to an extent that their mechanisms of interaction with algal cells and thereby bioavailability will be different from what could be expected from NPs in a simple control medium. The experimental process and methodology was tailored i) to achieve higher resolution in the quantification of Ag bioaccumulation to ensure that experimental bias was minimized and ii) to develop an approach by which the bioavailability of Ag from $AgNO_3$ and Ag NP sources in simple and complex media could be reasonably compared. Nevertheless, some of the challenges still remain in regard to the bioavailability of NPs in the environment. One of these challenges is that filter-based approaches used in the evaluation of NP bioavailability are limited by possible significant adsorptive losses on the filter membranes. Adsorptive losses limit our capacity to

observe bioaccumulation with lower NP (or dissolved Ag) exposure concentrations (less than 10 ug L^{-1}). Therefore, it is necessary to develop a methodology that would allow the detection and discrimination of cell-internalized Ag (NP or dissolved) without concession on the exposure concentrations. Also, NPs likely travel through urban sewage systems before reaching WWTPs, where they go through different stages of WW treatment process and eventually a fraction of NPs find their way to wastewater effluents. The variety and intensity of environmental factors acting on the NPs during different stages of their travel will produce NPs of different physicochemical properties. In a laboratory setting, however, it is challenging to simulate environmentally relevant conditions for NPs. Therefore, the careful extraction or separation of NPs in the environment for bioexposure experiments while keeping them physicochemically intact would be key to an environmentally relevant experiment. Also, for a successful bioexposure experiment, an important prerequisite is the capacity for detection and careful characterization of NPs in complex environmental media. Furthermore, it is essential to investigate possible interaction (bioavailability) scenarios between NPs/transformed species and cells that do not involve biointernalization but may lead to toxic effects. This necessity is prompted by the previous claims that toxic outcome upon exposure to NPs does not necessarily require the internalization of NPs (or relevant species).