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AgNPs break the wall cell in Chlorella vulgaris by oxidative stress generation

AgNPs rompe la pared celular en Clorella vulgaris mediante generación de estrés oxidativo

Reyes Reyes, A.K [1], Ocampo Fletes, I. [1], Ramírez Valverde, B [1], Ortiz Torres, E. [1], Sánchez Morales, P.[2], Acosta Mireles, M.[3]

[1] Laboratorio de Ecosistemas marinos, CUCBA, Universidad de Guadalajara, Camino Ramón Padilla Sánchez 2100, Código Postal 45200, Nextipac, Zapopan, Jalisco México. [2]Laboratorio de Patología, Departamento de Microbiología y Patología, CUCS, Universidad de Guadalajara, Sierra Mojada 950, Col. Independencia, Código Postal 44340, Guadalajara, Jalisco, México. [3]Centrao de Investigación en Nanociencias y Nanotecnología, Departamento de Ciencias Naturales y Exactas, CUValles, Universidad de Guadalajara, Carr. Guadalajara-Ameca Km45.5, Código Postal 46600, Ameca, Jalisco,México.

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ABSTRACT

Silver nanoparticles (AgNPs) are widely used due to their antibacterial activity. However, due to their nanometric size, they cannot be retained by wastewater filters and thus reach aquatic environments, which could affect microorganisms in the initial food chains, such as microalgae. This study aimed to elucidate the cytotoxic effects of AgNPs (3-7 nm) on Chlorella vulgaris ex situ. The AgNP synthesis was carried out through chemical reduction of silver nitrate and characterized by Transmission Electron Microscopy. C. vulgaris, collected from Chapala Lake, Jalisco, Mexico, was cultured in Bristol medium and exposed to different concentrations of silver nanoparticles (0.01, 0.1, and 1 mg/L) for 24 hours. A significant cytotoxic effect was observed in C. vulgaris exposed to silver nanoparticles, manifested by a decrease in chlorophyll-a content, morphological changes, prominent perforations in cell walls followed by Scanning Electron Microscopy (SEM), a significant lipid content reduction, and generation of oxidative stress, corresponding to the concentration of nanoparticles.

KEY WORDS: Silver; Nanoparticles; Chlorella vulgaris; cytotoxicity; oxidative-stress.

***Corresponding Author:**

Dra. en C. Irinea Yañez-Sánchez, Departamento de Ciencias Naturales y Exactas, CUValles, Universidad de Guadalajara, Carr. Guadalajara-Ameca Km 45.5, Código Postal 46600, Ameca, Jalisco, México; Teléfono: +52 (375) 7580 500. E-mail: irinea.yanez@academicos.udg.mx

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RESUMEN

Las nanopartículas de plata se utilizan ampliamente, en parte, por su actividad antibacteriana. Sin embargo, debido a su tamaño nanométrico, éstas no pueden ser retenidas por los filtros de aguas residuales por ende llegan al medio acuático; lo que podría afectar a microorganismos de las cadenas alimentarias iniciales como las microalgas. El propósito de este estudio fue dilucidar los efectos citotóxicos de las AgNPs (3-7 nm) en Chlorella vulgaris ex situ. La síntesis de nanopartículas de plata se realizó mediante reducción química de nitrato de plata; se caracterizaron por Microscopía Electrónica de Transmisión. C. vulgaris, recolectada del lago de Chapala, Jalisco, México, fue cultivada en medio Bristol expuestas a diferentes concentraciones de nanopartículas de plata (0.01, 0.1 y 1 mg/L) durante 24 horas. Se determinó un importante efecto citotóxico en C. vulgaris expuestas a las nanopartículas de plata, manifestado por disminución en el contenido de Clorofila a, cambios morfológicos, perforaciones prominentes en las paredes celulares mediante microscopía electrónica de barrido, disminución importante del contenido de lípidos y generación de estrés oxidativo, correspondiente a la concentración de nanopartículas.

PALABRAS CLAVE: Plata; Nanopartículas; Clorella-vulgaris; citotoxicidad, Estrés oxidativo.

Introduction

Advancements in nanotechnology have led to an increase in the application of nanomaterials across all fields of science and technology, primarily due to their physicochemical properties. As a result, the growing use of nanoparticles leads to their release into the environment, where they can potentially cause harmful effects. Metallic nanoparticles are ordered clusters of atoms with diameters ranging from 1 to 100 nm. Currently, there is a high demand for nanomaterials used in various products, with silver nanoparticles (AgNPs) being widely applied due to their potential antibacterial properties (Helmlinger et al., 2016). These applications include the detergent, cosmetics, pharmaceutical, food, electronics, textile, and paint industries. Additionally, these nanomaterials have been employed in agricultural activities as pesticides or fertilizers (Anand & Bhagat, 2019). This situation has led to the availability of around 1,300 products associated with nanoparticles (24 % of which contain AgNPs). The Environmental Protection Agency (EPA) indicates that 50 % of registered biocidal products containing silver likely contain AgNPs. Moreover, around 230 tons of AgNPs are produced annually in European countries (Pham, 2019). In 2012,

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the global production of AgNPs was approximately 60 tons, and it is expected to reach 900 tons by 2025 (Zhou et al., 2023). The AgNP concentration in water is approximately in the 1.27 to 2.89 µg/L range (Cao et al., 2019).

Regarding the antimicrobial activity of AgNPs, several mechanisms of action have been proposed. For example, the generation of free radicals can disrupt biological structures such as cell membranes, nucleic acids, and proteins. Another mechanism involves the binding of nanoparticles or silver ions to thiol groups in proteins, compromising their activity. Additionally, the chelation of micronutrients like phosphorus and carbon can alter membrane synthesis. These actions depend on the presence of Ag+ or AgNPs, and it has been shown that ionization depends on the oxygen presence, with smaller nanoparticles being more susceptible to ionization than larger ones (Helmlinger et al., 2016; Meroni et al., 2020; Nguyen et al., 2021; Pal et al., 2007).

The high demand for the production and disposal of nanoparticles, along with their large surface area and mobility, has increased interest in understanding their behavior and impact on the natural environment, particularly their interaction with and toxicity to aquatic organisms (Courtois et al., 2019; Giese et al., 2018). Several studies have shown that the intrinsic toxicity of AgNPs depends on various factors, including size, shape, surface area, surface charge, solubility, and aggregation state, among others (Romero et al., 2020).

As a primary producer, algae play a crucial role in maintaining the environmental balance of water bodies. In particular, green algae, or chlorophytes, are an important component of these aquatic ecosystems, with one of the most representative microorganisms being Chlorella vulgaris, which is the most abundant and commercially significant in health or as a fuel source (Deng et al., 2022). In addition to being one of the most representative species and indicators of damage to aquatic ecosystems (Choi & Hu, 2008; Das et al., 2014), microalgae can also be a significant source of natural antioxidants, offering an alternative to higher plants for the production of various chemical components beneficial to human health (Das et al., 2014; Dash et al., 2012; Hernández-Pérez & Labbé, 2014; Hiriart-Baer et al., 2006).The objective of this study was to determine the morphological and oxidative alterations of Chlorella vulgaris exposed to AgNPs.

Material and Methods

Synthesis and characterization of AgNPs

Silver nanoparticles were synthesized by dissolving $AgNO_3$ (0.1 g) in 100 mL of ethanol, using 1 g of polyvinylpyrrolidone (PVP) as a stabilizer (1:10), along with an equivalent amount of magnesium metal shavings. The solution was refluxed at 363 K with stirring for 12 hours. The characterization was performed using Ultraviolet-Visible (UV-Vis) spectrophotometry on a Varian® Cary 300 spectrophotometer with a spectral range of 200-900 nm. For High-Resolution Transmission Electron Microscopy (HR-TEM) analysis, samples (0.5 mL of a $5.224x10^{-3}$ M Ag° solution) were dissolved in concentrated isopropanol (2 mL), then deposited onto 300-mesh grids

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and dried. Finally, the samples were analyzed by HR-TEM using a TECNAI F30 microscope operating at 3000 kV.

Chlorella vulgaris culture

C. vulgaris was obtained from Chapala Lake, Jalisco, Mexico, using a plankton net at a depth of one meter. The algae were then isolated through serial cultures and maintained under laboratory conditions with continuous white light and a temperature of 25±1°C, using Bristol broth as the culture medium. Once exponential growth was reached (approximately $1x10^6$ cells/mL), *C. vulgaris* was subjected to treatments: a negative control and treatments with AgNPs at 0.01, 0.1, and 1.0 mg/L concentrations. The cultures were incubated under laboratory environmental conditions with constant stirring (120 rpm). The experiments were conducted in triplicate for 24 hours to determine the alterations in *C. vulgaris* due to AgNP exposure. Alongside, in all treatments, cell density was determined through microscopic counting at 24, 48, 72, and 96 hours using a Neubauer chamber. The growth kinetic parameters were calculated from the cell density.

Chlorophyll-α determination

The chlorophyll-α determination was performed on samples filtered using a GF/F filter with 45 µm pores and 25 mm diameter. The filter was kept refrigerated and protected from light in tubes containing 90 % acetone for extraction. The samples were then centrifuged at 4500 rpm for 15 minutes, and finally, readings were taken at 664, 647, and 630 nm using a Hach DR 2010 spectrophotometer.

Morphological alterations

For SEM analysis, the samples were centrifuged at 1000 rpm for 10 minutes to remove the culture medium and were rinsed with low-salt water. The samples were then fixed in a 2.5 % glutaraldehyde solution for 2 hours. They were centrifuged again for 1 minute at 1000 rpm to remove the fixative solution and washed twice with distilled water. The sediments were divided into small aliquots and placed on carbon tape and a silica gel chamber. These were kept in a silica gel drying chamber, and finally, the samples were coated with gold (99.9 % purity) using the sputtering technique. The samples were observed and analyzed using a JSM-6610 LV scanning electron microscope (Jeol®). To determine the size and morphological changes, approximately 50 cells from each treatment, were selected.

Oxidative stress and Neutral lipids content

The ROS determination was performed using the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA). Briefly, algal cells (10 mL) were washed by centrifugation; the sediment from each treatment was mixed in 100 µL of Bristol broth. Then, 250 µL of the reaction mixture containing the H_2DCFDA probe, ethanol, and PBS (10 µM, 10 µL, and 10 mL, respectively) was added and incubated for 1 hour at 25°C. Fluorescence was measured at an excitation wavelength of 450 nm and an emission wavelength of 595 nm using a Synergy HT

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microplate reader and Gen 5 2.0v software (Biotek®). To obtain the ROS formation index per cell, cells were counted by microscopic observation of live cells in a Neubauer chamber, and the ROS formation was divided by the number of cells.

The quantification of neutral lipids was carried with 3 µL of Nile Red stain (5 µM) those were mixed with 290 µL of DMSO (20 %) in 5 mL of cultured microalgae; the mixture was vortexed (120 rpm) and incubated at 40°C for 10 minutes. Then, 200 µL of this mixture was individually placed into a 96-well microplate. Fluorescence emissions were measured at excitation-emission wavelengths of 530-575 nm. This assay was performed in triplicate for each treatment (Chen et al., 2009; Elsey et al., 2007).

Statistical analysis

An ANOVA was performed to determine the significant differences ($p < 0.05$) in treatment groups of *C. vulgaris*. Statistical analysis was done through SPSS 20v software.

Results and Discussion

AgNPs characterization

The AgNPs synthesized showed a maximum absorbance peak at 420 nm during characterization by UV-visible spectrophotometry, corresponding to the presence of silver (data not shown). HR-TEM analysis determined that the nanoparticles were spherical, with sizes ranging from 3 to 10 nm, where the most representative sizes were 3 and 7 nanometers (Figure 1a, 1b).

The primary reason that AgNPs exhibit different properties compared to bulk materials and microparticles is due to their relatively large surface area to mass ratio, owing to the high particle number per unit mass in their preparations. This allows them to easily penetrate cell walls and alter internal biomolecules (Shanab et al., 2019). This condition could affect the chemical reactivity of biomolecules and may have harmful consequences for ecological communities. Standard safety regulations are controversial in some countries, and the ecological consequences of nanoparticle use have not been fully documented. Increasing amounts of AgNPs are expected to reach aquatic ecosystems, where their effects on natural phytoplankton communities are poorly understood, potentially posing risks to lower trophic levels and altering ecosystem functions (Deng et al., 2022).

It is widely known that nanoparticles smaller than 10 nm can directly alter cell permeability, enter bacterial cells, and cause damage. The influence of particle shape and size is described by the release of silver ions, as explained by the Ostwald-Freundlich equation, where small AgNPs with a spherical or quasi-spherical shape are more likely to release silver due to their greater surface area (Yin et al., 2020).

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Toxicity assay

In the present study, we showed the AgNPs toxic effects on the growth of C. vulgaris in a dose- and time-dependent; a potentially lethal effect on C. vulgaris when using AgNPs with a size of 7 nm and a concentration of 1 ppm (96 hours of exposure), with the complete death of the algae observed after 48 hours of exposure at a 10 ppm concentration has been reported (Phuong-Hong Lam, 2020). The growth kinetics of C. vulgaris exposed to AgNPs were significantly impacted depending on the exposure time, being more aggressive at a concentration of 0.1 mg/L (Figure 2a). The toxic effects of AgNPs on the growth of C. vulgaris were dose-dependent (Figure 2b), as determined by the chlorophyll-α pigment content, where a significant effect was observed after exposure to AgNPs, particularly at the two highest concentrations (0.1 and 1 mg/L, $p \leq 0.05$). Similarly, it was reported a strongly toxic effect, dependent on both dose and time, as reflected in the growth kinetics and cellular metabolism of C. vulgaris exposed to AgNPs (46.8 nm) at concentrations of 90-1440 µl/L (Romero et al., 2020).

Another study indicated that at concentrations higher than 12.5 µg/mL, toxic effects of AgNPs are observed, regardless of a particular morphology. Additionally, particles with a larger surface area (spherical) are more toxic to bacteria than to mesenchymal cells, suggesting that Ag+ is predominantly toxic in bacterial species (Helmlinger et al., 2016).

Similar decrements in chlorophyll-α content (51 %) after 24 hours of exposure to AgNPs with a diameter of 50 nm (10 mg/L) has been reported indicating a significant decrease of 8 %, 55 %, and 95 % (24 hours) in chlorophyll-α levels after treatment with 0.01, 0.1, and 1 mg/L of AgNPs (5 to 7 nm in size), respectively (Burczyk & Hesse, 1981; Oukarroum et al., 2012). Conversely, other studies have shown the effects of larger AgNPs (50 nm) on C. vulgaris, with results indicating a correlation between increased AgNP concentrations, sublethal physiological effects, and concentrations of 0, 10, 50, 100, and 200 mg/L over 8 days of exposure, which were higher than those used in this current study (Amal et al., 2013; Baky & El-Baroty, 2013). In a study the effects of AgNPs (10 nm size, 1, 5, and 10 µg/L) on phytoplankton growth (chlorophyll content) were examined at different phosphorus supply levels (over 72 hours). They reported a substantial decrease in chlorophyll levels related to increasing AgNPs concentrations and showed slow phytoplankton growth compared to a control group (Panahi et al., 2013). The most severe toxic effects observed in chlorophyll-α content in our current experiments (intermediate and high treatments) are mainly associated with the small size of the nanoparticles, as reported in similar studies (Ayyappan et al., 1997; Panahi et al., 2013; Perreault et al., 2012), but the toxic effect was less pronounced with larger nanoparticles (Burczyk & Hesse, 1981; Romero et al., 2020).

There is a close relationship between particle size and activity, as it depends on environmental conditions and the silver's ionization capacity, where smaller AgNPs ionize faster than larger ones, leading to higher toxicity of silver ions (Martínez-Castañón et al., 2008; Sotiriou & Pratsinis, 2011). On the other hand, spherical morphologies have biocompatibility and present less toxicity compared to rods or triangles in ocular cells, due to less genetic damage (presence of comet DNA), gradual ROS generation, and strong nanoparticle internalization (anisotropy) (Nguyen, 2021). With these insights, we can state that nanospheres can attach to the microalgae

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membrane and gradually penetrate the cells, not by “striking” but by generating pores and causing an imbalance in the membrane, which could induce the release of chlorophyll α and subsequently lead to cell death.

Morphological alterations

C. vulgaris can grow under heterotrophic and mixotrophic conditions, exhibiting spheroidal cell morphologies with diameters ranging from 2 to 15 µm (Romero et al., 2020). In the SEM analysis, the control algae cells showed an approximate size of 7.9 µm, with relatively round shapes and smooth surfaces (Figure 3). Regarding cell size, a significant difference was observed during the treatments, particularly at a concentration of 0.1 mg/L (size 7.0 µm). The silver nanoparticles had severe impacts on the cell wall, of microalgae with a significant presence of pores on the cell surface after exposure to the nanoparticles. Interestingly, the higher concentrations of AgNPs (0.1 and 1 mg/L) showed the most noticeable harmful effects on the cells, manifested by prominent grooves and highly distorted morphology.

In the SEM analysis, several significant damages to the C. vulgaris morphology were observed, similar to those reported in other studies performed on filamentous Chlorophyta (Seyfabadi et al., 2010). Similar alterations like our (cell wall perforations) has been reported, when exposing strains of C. pyrenoidosa and Daphnia magna to malachite green (a dye used in the food industry) and detected significant changes at the genetic level (Kanhere et al., 2014). The rigidity of the cell wall preserves the cell’s integrity and may confer an evolutionary advantage by offering stable protection against invaders and hostile environments. The cell wall varies in each growth phase according to environmental conditions; daughter cells contain a microfibrillar layer similar to chitosan composed of glucosamine (which explains its rigidity) and the presence of a sporopollenin layer (Das et al., 2014; Turner et al., 2012; van Beelen et al., 2009). The toxic damage to C. vulgaris could be the result of the accumulation of AgNPs within the cells, attachment to the cell walls, the physical protection of light necessary for algal growth by the nanoparticles (coating the surface), which hinders photosynthesis, or chelating various elements like P and C, needed for the C. vulgaris growth, especially for the chloroplast membranes formation that consist of a phospholipid bilayer (Das et al., 2014).

Some of the proposed mechanisms to explain membrane rupture, highlighting seven: a) rupture of the bacterial wall and membrane by silver ions (Ag+); b) denaturation of ribosomes; c) disruption of ATP production; d) membrane rupture due to ROS production; e) interference with DNA replication; f) membrane denaturation by AgNPs; and g) membrane perforation by AgNPs; which can be explained by the adhesion of AgNPs that penetrate the cytoplasmic membrane, generating the release or exit of organelles and eventually leading to cell lysis (Yin, 2020). In our results (Figure 3), we can observe this latter mechanism, through the generation of “pores” that may be caused by the presence of AgNPs adhered to the microalgae membrane.

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Oxidative stress and neutral lipids content

Currently, oxidative stress is believed to be the primary mechanism of toxicity for engineered nanomaterials. In our study, the ROS/cell number index increased dose-dependently in response to AgNP exposure relative to the cell number (Figure 4a). These results show a significant manifestation of free radical generation at 0.1 mg/L of AgNP exposure.

Nile Red staining is an excellent method for lipid determination in microalgae due to its speed, simplicity, and low biomass requirement. As a lipophilic dye, Nile Red has spectral properties determined by the polarity of its environment. It emits intense fluorescence in hydrophobic organic solvents and upon contact with lipid bodies. In our study, a significant (dose-dependent) decrease in neutral lipids was observed (Figure 4b) using AgNPs (3-7 nm in size).

As previously mentioned, oxidative stress is believed to be the primary mechanism of toxicity for engineered nanomaterials. Our analysis observed increases in ROS at all three concentrations, but the highest levels were at 0.1 mg/L of AgNP exposure. The mechanisms of this toxicity may, in turn, be influenced by particle size or ROS formation (Burczyk & Hesse, 1981; Rodríguez-García & Guil-Guerrero, 2008). The interaction with ROS could have played a role in the present study, as we incubated our treated cultures under natural light conditions. Conversely, it has reported that these changes in ROS could depend on the number of cells or chloroplasts (chlorophyll) and the esterase and peroxidase activities (Safi et al., 2014).

Humans obtain omega-3 polyunsaturated fatty acids (PUFA) through multiple levels of the food web: microalgae, which are used directly for food products or as animal feed; microalgae are the source of omega-3 PUFAs for various consumers, such as rotifers, copepods, Daphnia, and Artemia, which, in turn, serve as food for late larvae and juvenile fish and crustaceans (these fish then pass them on to consumers) (Kohen & Nyska, 2002; Lee, 2011); and livestock, which are fed with feed produced from various organisms in the food web.

As mentioned earlier, Nile Red staining is an effective method for determining microalgae lipids; in aqueous solutions, Nile Red fluorescence is completely quenched and can be easily quantified (Priyanka et al., 2020). A significant (dose-dependent) decrease in neutral lipids using AgNPs (3-7 nm in size), was observed. Contrary to our results, a paper reports a significant increase (87 % to 489 %) in total lipid content in microalgae exposed to AgNPs (but with a larger size, 46.8 nm) in a concentration-dependent manner (Romero et al., 2020). Similar results were reported that using 5 mg/L of AgNPs (6-12 nm in size), which promoted lipid content (14.3 %), but when the concentration of AgNPs was increased, lipid content decreased (Shanab et al., 2019).

These results could be influenced by various factors inherent to nanoparticles, such as shape, size, aggregation, or surface charge; similarly, the presence of oxygen influences the release of Ag+ ions. Several studies have indicated that the release of silver ions is more efficient in smaller silver particles, leading to greater toxicity. It has proposed an important role played by the adsorption of AgNPs (5 nm) and the silver ions released on the cell surface (synergy effects) (Zouzelka et al., 2016); the same occurs with specific surface area (Martínez-Castañón et

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al., 2008; Sotiriou & Pratsinis, 2011). There is a strong correlation between dissolution kinetics/ specific surface area in silver nanoparticles. Silver nanoparticles with a smaller specific surface dissolve less than those with a larger specific surface, suggesting that the oxidation of Ag to Ag+ on the particle surface is the rate-determining step in dissolution (Helmlinger et al., 2016).

Cytotoxicity of AgNPs/Clay in bacteria mediated by the cell wall was reported, stimulating the cytotoxic signal through a plate-like support and indicating that high concentrations of AgNPs in each silicate unit can alter membrane integrity, increase intracellular ROS production, and inactivate energy-dependent metabolism (Su, 2009). In our study, there was practically no significant difference in intracellular ROS generation (Figure 4); however, ROS production was sufficient to cause alterations in the membrane and interfere with ATP generation, leading to the chlorophyll-α efflux (Figure 2b).

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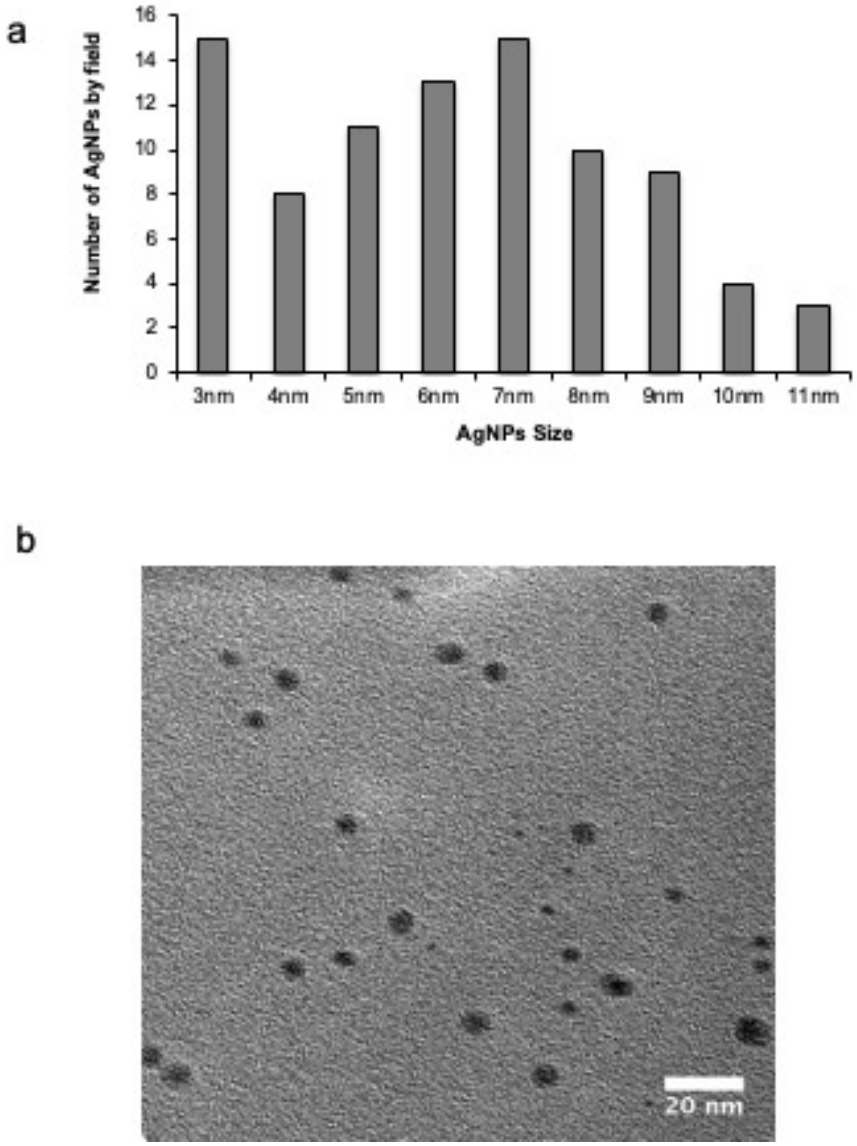

Figure 1. AgNPs characterization. In a) Particle size range, b) HR-TEM microphotography

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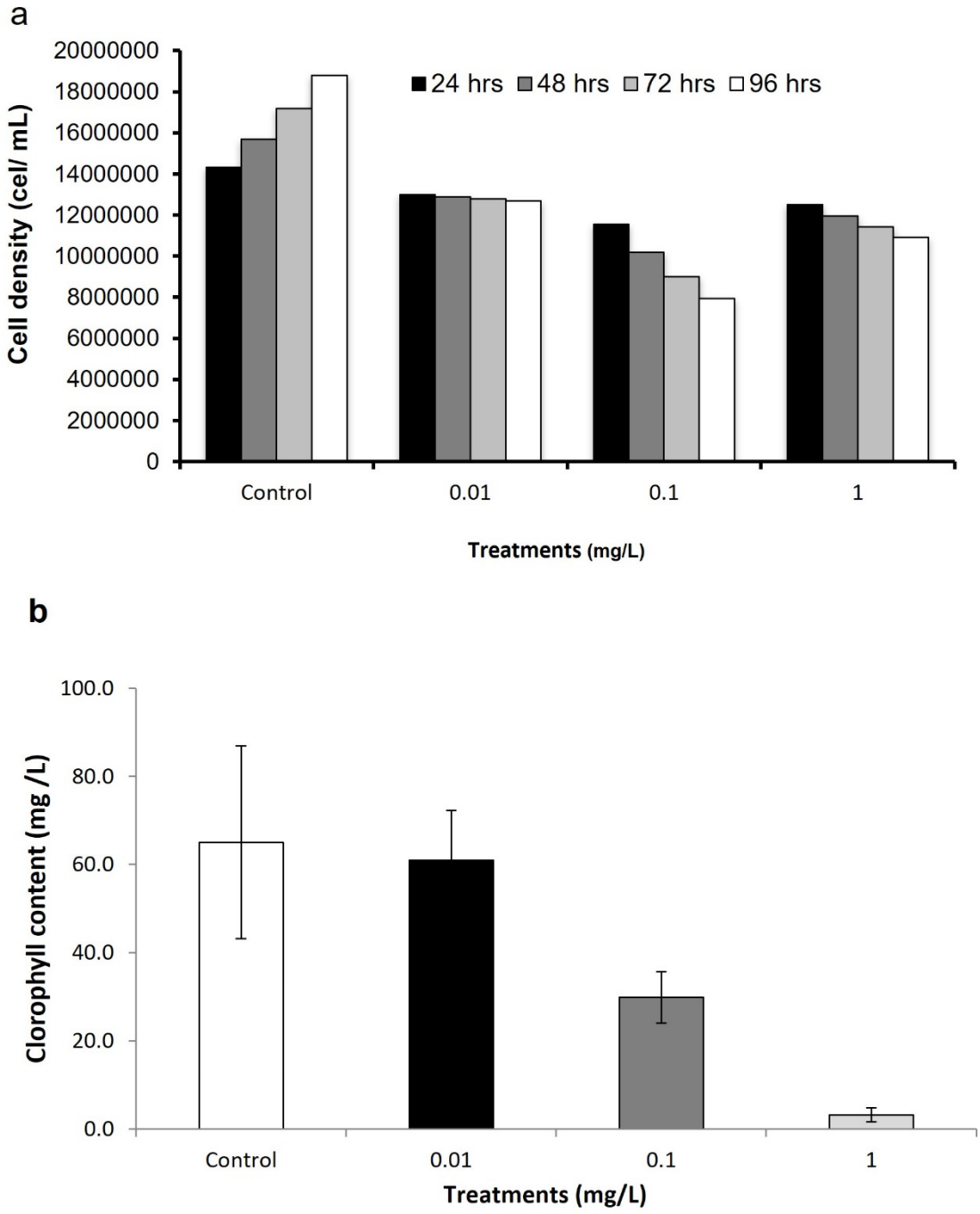

Figure 2. AgNPs toxicity in C. vulgaris. a) Proliferation assay of and b) Chlorophyll-a content in each experimental group.

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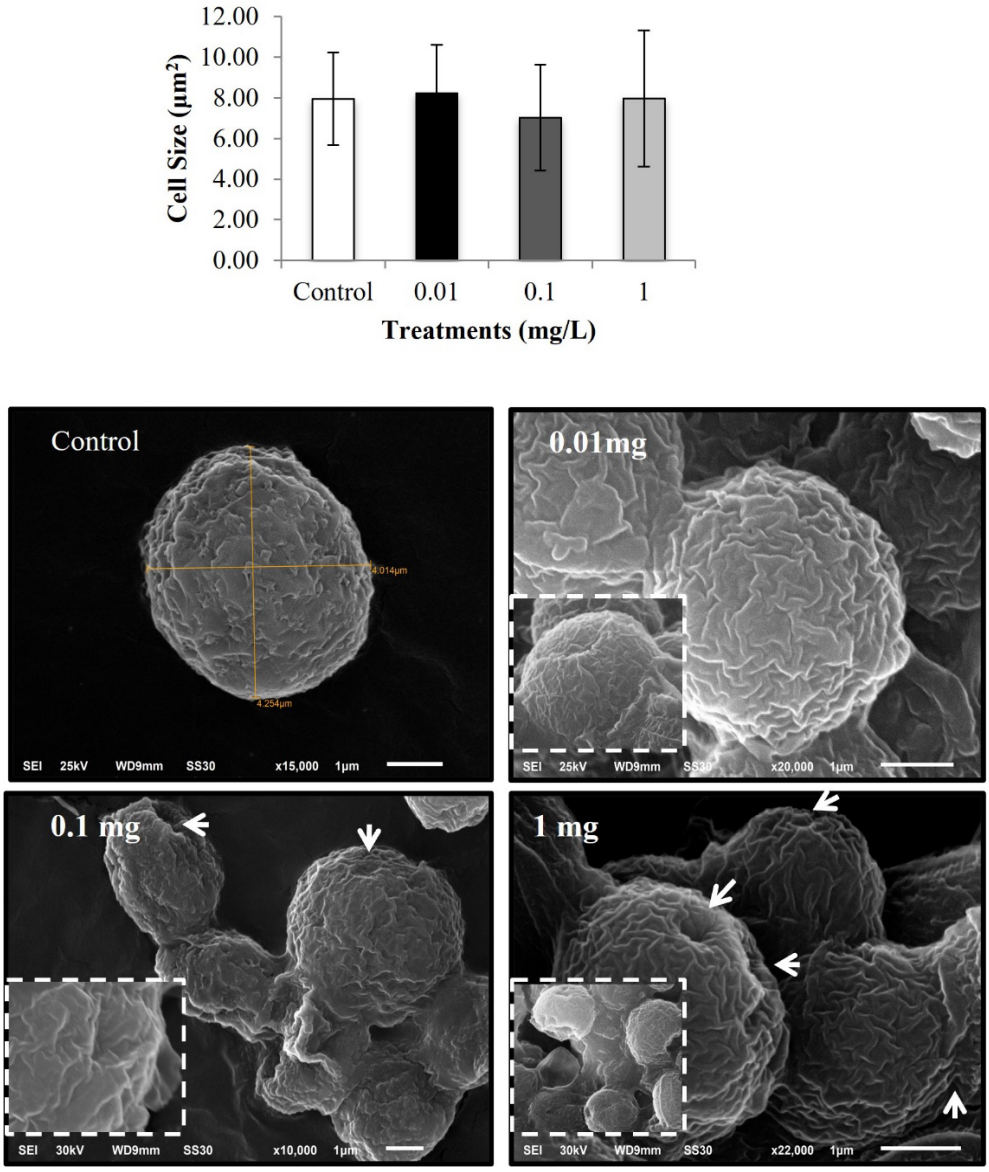

Figure 3. Morphologic alterations of *C. vulgaris*. SEM analysis of *C. vulgaris* exposed to AgNPs.

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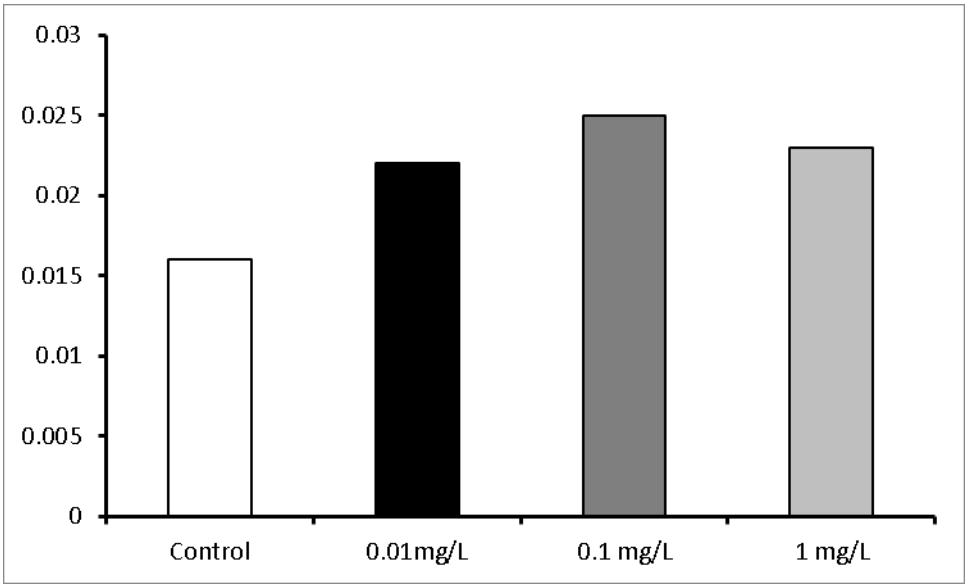

Figure 4. Biochemical impact of AgNPs. a) ROS/Cell number index and b) Neutral lipids content of *C. vulgaris* exposed to different concentrations of AgNPs.

Conclusions

We conclude that AgNPs accumulate in the cell wall of *C. vulgaris*. This accumulation of nanoparticles allows the release of Ag^{+} ions, inducing oxidative stress in the cell wall and producing a small pore, approximately 0.5 µm in diameter, in the cell wall, as shown in the SEM image. Finally, the effects of AgNPs on the microalga *C. vulgaris* non-specifically damage chlorophyll-a and the cell wall morphology. It is important to continue studying the effects of nanoparticles on organisms, as their behavior and bioavailability in ecosystems, as well as their persistence and potential impact due to bioaccumulation in food chains, remain unknown.

Authors Contributions

EGRJ, FJGG, IYS: Design, content definition, literature search; EGRJ, CVO: Methodology development; FJGG: Software; IYS, MLOM, FJGG: Experimental validation, data acquisition, and analysis; FJGG, IYS: Writing and editing of the manuscript, supervision; IYS, CVO: Resources,

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project administration. All authors of this manuscript have read and approved the published version.

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Conflict of Interest

The authors declare no conflict of interest.

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