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Glucose-mixotrophic culture of the freshwater alga *Chlorella vulgaris* alleviates the phytotoxicity of silver nanoparticles

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Running title: GLUCOSE REDUCES AgNP TOXICITY IN *CHLORELLA VULGARIS*

Abstract – Silver nanoparticles (AgNPs) are widely used due to their specific physico-chemical properties, but their release in wastewater poses a threat to aquatic ecosystems. The microalga *Chlorella vulgaris* is often used as a model organism in toxicological research. Since this alga can switch from an autotrophic to a mixotrophic mode of life, we investigated whether glucose as an additional energy source can mitigate AgNP phytotoxic effects. Algae were cultivated in a nutrient medium with and without glucose and treated with a concentration of AgNPs allowing 60% cell survival. The effects were studied by monitoring oxidative stress and photosynthesis parameters. In the absence of glucose, AgNPs caused significant accumulation of reactive oxygen species, increased lipid peroxidation and carotenoid content with weak activation of antioxidant enzymes. In addition, reduced photosynthetic efficiency and ultrastructural damage were observed. The addition of glucose to AgNP treatments attenuated all negative effects by reducing oxidative stress and increasing the activity of antioxidant enzymes. Furthermore, *C. vulgaris* cells exposed in glucose-enriched media showed recovered photosynthesis by exhibiting the photosynthetic rate and chlorophyll fluorescence parameters of control algae. Ultrastructural analysis showed greater synthesis of lipid droplets and less structural damage. The study shows that glucose supplementation effectively mitigates the phytotoxic effects of AgNPs on *C. vulgaris*. By reducing oxidative stress, enhancing antioxidant enzyme activity and restoring photosynthetic function, glucose helps maintain cellular integrity and overall algal health in the presence of AgNPs, which suggests that mixotrophic cultivation may represent a protective strategy against nanoparticle-induced toxicity in aquatic microalgae.

Keywords: antioxidant enzymes, freshwater alga, mixotrophy, photosynthetic pigments, photosystem II, reactive oxygen species, ultrastructure

Introduction

Nanotechnology is a branch of science that modulates materials at the atomic and molecular level in the range of 0 to 100 nm. When the dimensions of a material are reduced to the nano level, there are significant changes in the physico-chemical properties, which consequently also change the properties of the material. These properties enable the use of nanomaterials in numerous areas, from medicine to the food industry (Anees Ahmad et al. 2020). Among metal nanoparticles, silver nanoparticles (AgNPs) are among the most widely used, mainly due to their unique properties such as their exceptional optical properties, electrical and thermal

conductivity, but also because of their biological properties, which is why they have applications in medicine, in commercially available products, in the food industry and, increasingly, in diagnostics, drug delivery and as anti-cancer agents (Zeng et al. 2024). Their primary biological activity is based on their biocidal effect, which is achieved through the gradual release of silver ions (Ag^+) that can act on cells via various mechanisms by interfering with the thiol groups of proteins, inhibiting DNA replication and triggering oxidative stress (Anees Ahmad et al. 2020). With the increasing annual production and use of AgNPs, their release into the environment is also increasing, which means that they inevitably enter aquatic ecosystems via wastewater and rainwater, where microalgae are usually the dominant organisms (Zeng et al. 2024).

Microalgae are a diverse group of unicellular autotrophic organisms. Apart from the fact that microalgae, together with cyanobacteria, produce about 50% of the world's oxygen through the process of photosynthesis, they are also an excellent source of carbohydrates, proteins, lipids, nutrients, vitamins and minerals (Roth et al. 2019). For this reason, they are often used as food supplements, as well as in the pharmaceutical industry, in dye production, as animal feed, in aquaculture and in cosmetics (Castillo et al. 2021). *Chlorella vulgaris* is one of the best-known species of microalgae and is found in freshwater and in the sea. It is known for its high photosynthetic rate and rapid growth, especially under mixotrophic and heterotrophic conditions. Its ability to adapt to different conditions enables a wide range of applications in industry, but also makes it an excellent biosensor for environmental pollutants. In addition, *C. vulgaris* produces primary metabolites required for growth and maintenance of basic metabolic processes, while secondary metabolites are synthesized in response to stress conditions, further increasing its applicability in biotechnology (Ru et al. 2020).

Although microalgae are photosynthetic organisms, many species show the ability to switch to mixotrophic or heterotrophic conditions. Mixotrophic microalga growth is based on the addition of organic carbon sources (i. e. carbohydrates, organic acids, alcohols, amino acids) in the presence of light. Mixotrophic cultivation enables the production of valuable compounds such as fatty acids and polysaccharides and offers advantages over autotrophy such as faster growth and improved efficiency (Castillo et al. 2021). Glucose, the most commonly used carbon source in mixotrophic cultures, allows algae to utilize light energy and organic matter simultaneously. A key advantage of mixotrophic cultivation is the ability to adjust conditions to achieve an optimal balance between photosynthesis and the direct use of glucose as a carbon source (Ru et al. 2020). This not only accelerates algal growth but also allows precise control of the composition of metabolites, which is crucial for certain industrial applications such as food production or the synthesis of pharmaceutical compounds. Therefore, the use of glucose in mixotrophic cultures represents a promising approach to optimize microalgae cultivation, especially in situations where conditions for photosynthesis are suboptimal or when the goal is to achieve maximum biomass production in a shorter period of time (Castillo et al. 2021).

Previous studies have shown that the addition of glucose can have a significant effect on the photosynthetic apparatus of microalgae. Positive effects were observed in cultures of *Chlorella sorokiniana*, where the addition of glucose resulted in higher photosynthetic efficiency and a higher relative electron transport rate, which in turn led to biochemical changes in terms of protein and lipid content (Marchello et al. 2018). However, *Chromochloris zofingiensis* was shown to reversibly shut down photosynthesis in the presence of glucose (Roth et al. 2019), while in *Chlorella kessleri* the addition of glucose reduced chlorophyll content (Deng et al. 2019). Furthermore, higher glucose concentrations (> 3% w/v) were shown to inhibit chloroplast development in *Arabidopsis* seedlings by suppressing certain photosynthetic genes (To et al. 2003) and reducing the photosynthetic rate and electron transport (Eckstein et al. 2012).

In addition to the effects on the photosynthetic apparatus, the addition of glucose as an additional carbon source can also mitigate the harmful effects of abiotic stress, as in the reduction of cadmium toxicity (Huang et al. 2024), and the reduction of nonylphenol toxicity in *Chlorella pyrenoidosa* (Yuan et al. 2025), the reduction of cadmium and copper toxicity in *Arabidopsis thaliana* (Shi et al. 2015) and *Cucumis sativus* (Yusuf et al. 2021) and the improvement of salt stress tolerance in *C. sativus* seedlings (Ma et al. 2020). In addition, studies have shown that glucose improves the tolerance of various microalgae species to high ammonia levels (Sutherland, 2022). However, the effects of glucose addition on AgNP toxicity have been very poorly investigated. A study on the phytotoxicity of AgNPs to human liver cells HepG2 has shown that glucose has a mitigating effect by reducing the cytotoxic effects of AgNPs mainly by increasing antioxidant defence and reducing the production of reactive oxygen species (ROS) (Zuberek et al. 2015). Moreover, another study showed that a high-carbohydrate diet can attenuate oxidative stress, inflammation and apoptosis induced by AgNPs in *Megalobrama amblycephala* (Chen et al. 2021). Although sparse, these data suggest that glucose may reduce AgNP-induced toxicity by mitigating oxidative stress and modulating various cellular processes.

Therefore, in this study we investigated whether the phytotoxic effects of AgNPs on the freshwater green alga *C. vulgaris* grown under autotrophic conditions can be mitigated by the addition of 0.5% glucose to the nutrient medium. To ensure comparability, the effective concentration of AgNPs allowing 60% cell survival (EC_{40}) was first determined in cultures grown without glucose (-Glu). This concentration was then applied to both groups of algae, cultivated in parallel: one in medium without glucose (-Glu) and the other in medium with glucose (+Glu), to evaluate whether glucose supplementation can attenuate AgNP-induced toxicity. During the study, we analysed the accumulation of silver, the occurrence and attenuation of oxidative stress, the effects on the photosynthetic apparatus and the ultrastructural changes in the cells.

Material and methods

AgNP synthesis and characterization

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical grade unless otherwise stated, while ultrapure water (Milli-Q, 18.2 MΩ-cm, Merck Millipore, Billerica, MA, USA) was used for all syntheses.

The synthesis of citrate-stabilised AgNPs (AgNP-citrate) was carried out according to the method of Peharec Štefanić et al. (2021). To 120 mL of boiling $AgNO_3$ solution (0.02 g), 5 mL of sodium citrate solution (1% *w/v*) was added. A colour change from clear to pale yellow signalled the formation of AgNPs, whereupon the suspension was rapidly cooled to room temperature and stored at 4 °C until analysis. The physical and chemical properties of the AgNP-citrate stock suspension were analysed according to previously published procedures (Peharec Štefanić et al. 2021). The presence of AgNPs was confirmed by observing a characteristic surface plasmon resonance (SPR) peak using a UV-Vis spectrophotometer (Unicam, Cheshire, UK). The hydrodynamic diameter (d_H) and ζ-potential were measured by dynamic light scattering (DLS) and electrophoretic light scattering (ELS) using a NanoBrook 90Plus particle size analyser (Brookhaven Instruments, Holtsville, NY, USA), while the data were processed using Zeta Plus 5.71 software. The amount of Ag^+ ions released by dissolving AgNPs in ultrapure water was determined by centrifugal ultrafiltration (Millipore Amicon Ultra-4 3K, with 3 kDa membrane) and by an inductively coupled plasma mass spectrometer (ICP-MS) (ELAN DRC-e, Perkin Elmer, Waltham, MA, USA) using a calibration curve with standards; the detection and quantification limits were 0.2 mg kg^{-1} and 1 mg kg^{-1}, respectively. Finally, the synthesised and purified AgNP-citrate was visualised

using a TEM (TF20, FEI Tecnai G2, equipped with an EDX detector, FEI, Hillsboro, OR, USA) according to the method described in Peharec Štefanić et al. (2021).

Chlorella vulgaris cell culture

C. vulgaris cultures were grown in liquid BBM medium (Nichols and Bold, 1965) in autoclaved 200 mL Erlenmeyer flasks. Two versions of the medium were used for control purposes: BBM without glucose (-Glu) and BBM with 0.5% (w/v) glucose (+Glu). Cultivation was carried out in their corresponding media (-Glu and +Glu) throughout the experiment in a plant growth chamber at 24 °C under long-day conditions (16 h of light, 8 h of darkness) and a light intensity of 80 µmol m^{-2} s^{-1}, with constant orbital movement.

The growth of the culture was monitored for 30 days by daily cell counts using an automated cell counter (Luna II, Logos Biosystems, Anyang, Republic of Korea), which determined the exponential growth phase in the media -Glu and +Glu. To enable a direct comparison between treatments, cultures (-Glu and +Glu) were inoculated with different initial cell concentrations so that, after four days of cultivation, both control groups reached the same cell concentration of 1×10^6 cells mL^{-1}. At this point, both cultures were treated with 0.251 mg L^{-1} AgNP-citrate, corresponding to the EC_{40} value previously established in -Glu medium, i.e. the concentration that ensured 60% cell survival in -Glu medium.

Cell viability test using flow cytometry

To determine the EC_{40} value, a culture with an initial concentration of 1×10^5 cells mL^{-1} was prepared by inoculation in liquid -Glu medium in sterile Erlenmeyer flasks. The algae were then grown for four days under the indicated conditions with constant orbital mixing until a concentration of 1×10^6 cells mL^{-1} was reached in the exponential phase. According to OECD guidelines, these cultures were exposed to AgNP-citrate concentrations between 0.005 and 3.5 mg L^{-1} for 72 h.

The EC_{40} concentration after 72 h of treatment in -Glu medium was determined by cell survival analysis using flow cytometry in combination with the fluorescent dye propidium iodide (PI), as previously reported in Qi et al. (2022). Briefly, 1 mL of cell suspension was mixed with PI at a final concentration of 1 µg mL^{-1}, samples were incubated in the dark for 5 min, and then analysed using a flow cytometer (BD FACSVerseTM, Piscatway, NJ, USA) with a 488-nm laser. In all, 100,000 cells per sample were analysed, and the concentration leading to EC_{40} was determined using the four-parameter logistic (4PL) regression model (Dawson et al. 2012). The algal cultures without the addition of AgNP-citrate served as control samples for their corresponding media (Control (-Glu) and Control (+Glu)), and each experiment was performed in triplicate. Silver content, oxidative stress levels, antioxidant response parameters, as well as ultrastructural and photosynthetic activity analyses were determined in algal cells grown in -Glu and +Glu media. These cells were first cultivated for 4 days with initial cell concentrations adjusted to reach 1×10^6 cells mL^{-1} and then exposed for 72 h to 0.251 mg L^{-1} of AgNP-citrate. The results were compared with their corresponding control groups.

Measurements of the silver content

The silver content in algal cells was measured according to the previously described method (Komazec et al. 2023). The cell culture was centrifuged at 3500 × g at room temperature for 5 min, and the resulting pellet was washed three times with ultrapure water. After each wash, the cells were centrifuged again under the same conditions, and the resulting pellet was freeze-dried for 24 h. The cells from the pellet were digested in a microwave oven (ETHOS SEL Milestone, Shelton, CT, USA) according to EPA method 3051a. In the first step, digestion was carried out in 10 mL of concentrated nitric acid (HNO_3) at 130 °C for 10

min and then at 180 °C for a further 15 min. In the next step, digestion was carried out in 1 mL of 30% hydrogen peroxide (H_2O_2) at 85 °C for 5 min and continued at 130 °C for 4 min. After cooling, the samples were diluted with 1% (v/v) HNO_3 to a final volume of 50 mL. The total silver content was determined with the ICP-MS instrument ELAN DRC-e, using a calibration curve from a series of standards with known concentrations for the calculation. The limits of detection and quantification (LOQ) were determined to be 0.05 mg kg^{-1} and 0.1 mg kg^{-1} and the percentage recoveries were 95.6%, 95.2% and 96.5%, respectively. The final results are expressed in micrograms of silver per 10^6 cells.

Analyses of oxidative stress parameters

Determination of reactive oxygen species

ROS content was determined using the fluorescent markers dihydroethidium (DHE) and 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) according to the adapted protocols described in Cvjetko et al. (2017) and Ng and Ooi (2021), respectively. DHE measures the superoxide anion ($O_2^{\bullet -}$), while H_2DCFDA measures the total ROS content. In a 96-well microplate, 80 µL of a 20 µM DHE or H_2DCFDA solution was added to each well containing 100 µL of cell suspension. Samples were then incubated in the dark for 30 min, after which fluorescence was measured using a GloMax microplate reader (Promega, Madison, WI, USA). An excitation wavelength of 520 nm and an emission wavelength of 600 nm were used for the DHE probe, while an excitation wavelength of 504 nm and an emission wavelength of 550 nm were used for H_2DCFDA. The results were normalized to the number of cells and expressed as a percentage of the ROS content in the control samples.

The H_2O_2 content was measured according to a modified protocol described in Mátai and Hideg (2017). For this purpose, 200 ml of cell suspension was centrifuged at 3500 × g for 5 min at 20 °C. After the supernatant was discarded, the cell pellet was washed three times with ultrapure water and centrifugation was repeated under the same conditions after each wash. The cell pellet was resuspended in cold 70% ethanol and homogenized in a Retsch homogenizer (MM200, Retsch, Haan, Germany) at a frequency of 30 Hz for 3 min at 4 °C with the addition of glass beads (425-600 µm). After homogenization, the samples were centrifuged at 15 000 × g for 10 min at 4 °C, and the supernatant was transferred to clean tubes and stored on ice. After extraction, 100 µL of the prepared sample was added to 1000 µL of a reaction mixture containing 124 µM xylenol orange (sodium salt), 99 mM sorbitol, and 0.248 mM ammonium iron (II) sulphate. After being briefly shaken, the mixture was incubated at room temperature for 15 min and the absorbance was measured at 560 nm. The total amount of H_2O_2 was determined by comparison with prepared standard solutions, and the results were expressed as µmol H_2O_2 per 10^6 cells.

Level of lipid peroxidation

The intensity of lipid peroxidation was determined by quantification of malondialdehyde (MDA) using an adapted protocol according to Heath and Packer (1968). First, 200 mL of the cell suspension was centrifuged at 3500 × g for 5 min at 20 °C. The precipitate was then washed three times with ultrapure water and centrifuged again under the same conditions. Then, the cell pellet was homogenized in three cycles using a MM200 Retsch homogenizer at 30 Hz for 3 min, with the addition of glass beads (425-600 µm) to 700 µL of a solution containing 0.5% (w/v) 2-thiobarbituric acid (prepared in 20 % (w/v) trichloroacetic acid). The resulting extracts were incubated at 95 °C for 30 min and then centrifuged at 14 000 × g min at 4 °C for 30. Absorbance was measured at 532 nm, with the value measured at 600 nm subtracted to correct for non-specific turbidity. Final calculations of MDA content

were normalized to the number of cells in each sample and expressed as a percentage of control cells.

Protein extraction

To prepare the protein extracts, 200 mL of cell suspension was centrifuged at 3500 × g for 5 min at 20 °C. After the supernatant was discarded, the remaining pellet was washed three times with ultrapure water, with an additional centrifugation (3500 × g, 20 °C, 3 min) after each wash. After the washing cycle, the cells were homogenized using a MM200 Retsch homogenizer at 30 Hz for 3 min at 4 °C in the presence of glass beads (425-600 µm) and 500 µL of potassium phosphate buffer (50 M, pH 7.0). The resulting homogenate was then centrifuged at 20 000 × g for 15 min at 4 °C, and the supernatant was transferred to clean tubes and centrifuged for another 45 min under the same conditions. Protein concentration was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin (BSA) as a standard. The extracts were then used for quantification of protein carbonyls and analysis of enzyme activity.

Activity assays of antioxidant enzymes

Enzyme kinetics were analysed at room temperature using a UV/Vis spectrophotometer (ATI UNICAM UV4, Cambridge, UK).

The activity of pyrogallol peroxidase (PPX, EC 1.11.1.7) was measured according to the protocol of Nakano and Asada (1981). The protein extract was added to the reaction mixture containing 50 mM potassium phosphate (pH 7.0), 20 mM pyrogallol and 5 mM H_2O_2, at a final concentration of 2 % (v/v). The oxidation of pyrogallol, which led to an increase in absorbance at 430 nm (extinction coefficient $\varepsilon = 2.6\ mM^{-1}\ cm^{-1}$), allowed the determination of the specific activity of PPX expressed as $\mu mol_{purpurogalin}\ min^{-1}\ mg^{-1}$ of protein.

The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was analysed using a modified version of the method of Nakano and Asada (1981). The protein extract was added to a reaction mixture of 50 mM potassium phosphate (pH 7.0), 0.5 mM ascorbate and 10 mM H_2O_2 at a final concentration of 18 % (v/v). The decrease in absorbance at 290 nm (extinction coefficient $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$) was used to calculate the specific APX activity, expressed as $\mu mol_{oxidized\ ascorbate}\ min^{-1}\ mg^{-1}$ protein.

Catalase activity (CAT, EC 1.11.1.6) was determined according to a modified procedure (Aebi, 1984). The protein extract was added to a reaction mixture of 50 mM potassium phosphate (pH 7.0) and 20 mM H_2O_2 at a concentration of 5 % (v/v). The decrease in absorbance at 240 nm (extinction coefficient $\varepsilon = 36\ mM^{-1}\ cm^{-1}$) was observed, and the results were expressed as $\mu mol_{degraded\ H2O2}\ min^{-1}\ mg^{-1}$ protein.

Analyses of photosynthesis parameters

Rate of photosynthesis

To determine the photosynthetic rate, the cell concentrations in all cultures were first measured using the Luna II device, while the photosynthetic rate was measured using the Chlorolab 2 device (Hansatech, King's Lynn, UK). A 1.5 mL sample of the algal culture was added to the reaction vessel of the device, and the amount of oxygen produced was monitored under continuous stirring at 30 rpm at a temperature of 30 °C and a light intensity of 80 µmol $m^{-2}\ s^{-1}$. Between periods of illumination, the algal suspensions were incubated in the dark for 5 min. The data obtained showed the amount of oxygen released, and the photosynthetic rate values were calculated per second and expressed as nmol of oxygen per hour per 10^6 algal cells.

Parameters of chlorophyll *a* fluorescence

To determine the maximum quantum yield (F_v/F_m) of photosystem II (PSII) and the efficiency index (PI_{abs}) of PSII, the cell concentrations in the algal suspensions were measured after treatment and compared to the corresponding untreated controls using a Luna II cell counter. The algal suspensions were then incubated in the dark for 1 h to allow oxidation of PSII and deactivation of non-photochemical quenching mechanisms. Samples were prepared in cuvettes of the AquaPen instrument (Photon Systems Instruments, Drásov, Czech Republic) with 3 mL of the suspension added to each cuvette. The fluorescence of Chl *a* was monitored with the OJIP test using a superpulse of 53 % strength. The results were read with the program FluorPen ver. 1.1.1.3. (Photon System Instruments, Drásov, Czech Republic) and expressed as the ratio between variable and maximum fluorescence, adjusted to dark conditions (F_v/F_m), and as arbitrary PSII efficiency units (PI_{abs}).

Content of the photosynthetic pigments

To determine the concentrations of the photosynthetic pigments Chl *a* and *b* as well as of carotenoids, the cell concentrations in the suspensions were first measured using a Luna II cell counter. Based on these concentrations, the appropriate volume of the cell suspensions, containing 7.5×10^6 cells was transferred to clean 1.5 mL tubes. Pigment isolation was performed according to a previously modified protocol (Schumann et al. 2005). Samples were centrifuged at 3500 × g for 5 min, then the supernatant was removed. The resulting pellet was washed 3 times with ultrapure water and centrifuged again under the same conditions after each wash. After the last wash, the pellet was resuspended in 800 µL of cold 90 % acetone and 0.083 g of silica beads (425-600 µm) were added. The cells were then homogenized in cold carriers on a MM200 Retsch homogenizer at 30 Hz for 4 min.

After homogenization, the samples were centrifuged again at 3500 × g at +4 °C for 5 min. The supernatant was transferred to a dark 2.0 mL tube and stored at +4 °C. The pellet was resuspended in 500 µL of cold 90 % acetone, homogenized and centrifuged again under the same conditions. The resulting supernatants were combined, and the pellet was resuspended a third time in 500 µL of cold acetone, homogenized and centrifuged again under the same conditions. All three supernatants were combined, and the volume was made up to 2 mL with cold 90 % acetone.

The absorbance of the samples was measured at the wavelengths of 647, 664 and 470 nm, and the concentrations of Chl *a* and *b* and carotenoids were calculated according to formulae described previously (Jeffrey and Humphrey, 1975; Dere et al. 1998). The results obtained are expressed as µg of Chl *a* or *b* per 10^6 cells, and µg of carotenoids per 10^6 cells.

Analyses of cell ultrastructure

Ultrastructural changes were analysed by transmission electron microscopy (TEM). Algal cells were first centrifuged at 3500 × g for 3 min at room temperature, after which the resulting pellet was fixed in 1 % (w/v) glutaraldehyde prepared in 50 mM cacodylate buffer (pH 7.2). Subsequently, 50 µL of the fixed cell suspension was transferred to clean tubes and mixed with liquid 1.5 % (w/v) agarose. The mixture was carefully resuspended, and after solidification, the separated agarose pieces with the cells were additionally fixed at 4 °C for 1.5 h. After fixation, the samples were washed twice with cold 50 mM cacodylate buffer (pH 7.2) and post-fixed with 1 % (w/v) osmium tetroxide in the same buffer at 4 °C for 1.5 h. This was followed by a double rinse with ultrapure water (10 min at 4 °C) before the samples were gradually dehydrated with ethanol. After the final dehydration step, the samples were embedded in Spurr resin. Ultrathin sections were stained with UranyLess (Em-Grade.com, Mauressac, France) and 3 % (w/v) Reynolds lead citrate (Em-Grade.com, Mauressac, France) and analysed using a monochromatic TF20 TEM (FEI Tecnai G2).

Statistical analysis

Statistical analysis of the data was performed using one-way ANOVA analysis followed by Newman-Keuls post-hoc analysis. The STATISTICA 14.0.0.15 software package (TIBCO Software Inc., Palo Alto, CA, USA) was used for the analysis, and statistically significant differences between the means values were determined at a significance level of $P \leq 0.05$.

Results

AgNPs physico-chemical properties

The AgNP stock suspension in ultrapure water was analysed by UV-Vis spectroscopy, TEM, DLS and ELS, and the results are presented in the Supplementary materials. Spectrophotometric analysis showed an SPR peak at 418 nm, indicating successful synthesis of AgNPs with a nominal diameter of 47 nm (On-line Suppl. Fig. 1A), while DLS analysis indicated an average d_H value of 41.4 ± 0.9 nm (On-line Suppl. Tab. 1). Furthermore, TEM micrographs revealed the spherical morphology of AgNPs with a diameter of 40–60 nm, with a small proportion of rod-like particles (On-line Suppl. Fig. 1B). The silver map (On-line Suppl. Fig. 1C) and energy dispersive X-ray microanalysis (On-line Suppl. Fig. 1D) confirmed the presence of silver in the particles. The AgNP ζ-potential was -40.50 ± 3.21 mV (On-line Suppl. Tab. 1). The silver concentration in the AgNP stock suspension measured by ICP-MS was 108.5 mg L^{-1} with 0.5% ionic silver (On-line Suppl. Tab. 1).

Cell viability response to AgNP exposure

Cell viability following 72 h exposure to varying concentrations of AgNPs in BBM medium is presented in Fig. 1. A clear concentration-dependent decrease in viability can be observed. The EC_{40} value, which indicates the concentration at which 60% of cells remained viable, was calculated using a four-parameter logistic (4PL) model. For the AgNP (-Glu) treatment, the EC_{40} was determined to be 0.251 mg L^{-1}, based on flow cytometry analysis with PI staining.

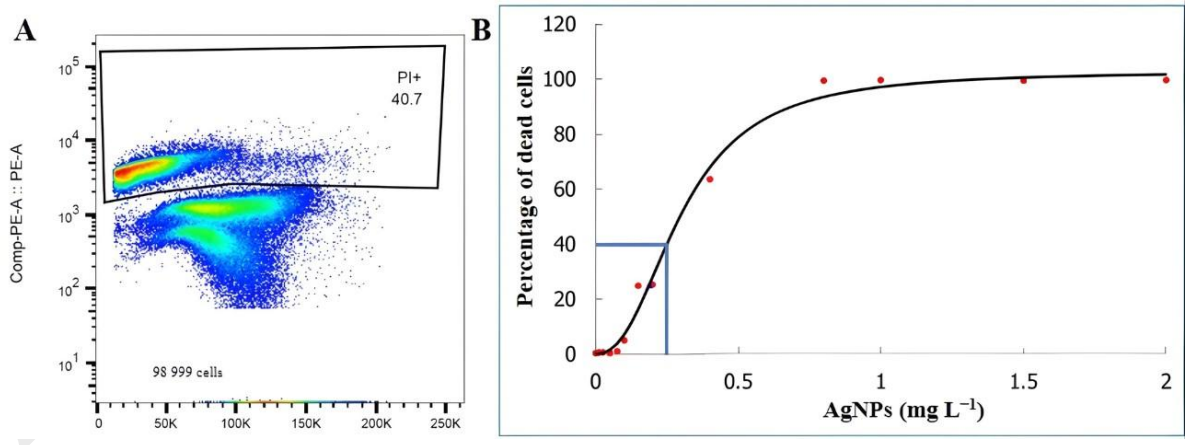

Fig. 1. The viability of *C. vulgaris* cells after 72 h exposure to increasing concentrations of AgNPs in BBM medium without glucose (-Glu) was determined by cell survival analysis using flow cytometry and the fluorescent dye propidium iodide. The results of flow cytometry (A) show approximately 40% dead cells. A four-parameter logistic (4PL) regression model was applied to determine the concentrations that caused EC_{40} (B), and the value obtained was used in all subsequent experiments. The values shown are the mean values of two independent experiments, each performed in triplicate (N = 6). The blue line indicates the EC_{40} value.

Silver uptake

Silver accumulation in algal cells after 72 h exposure to 0.251 mg L^{-1} AgNPs in BBM medium is summarized in Table 1. Detectable levels of silver were found in both treatment conditions, with significantly higher accumulation observed in the AgNP (-Glu) group (1234.10 ± 285.48 µg g^{-1} dry mass) compared to AgNP (+Glu) (404.31 ± 60.46 µg g^{-1} dry mass). In contrast, silver content in both control groups (-Glu and +Glu) remained below the detection limit. These results suggest that glucose reduces AgNP uptake in algal cells.

Tab. 1. Silver content (µg 10^{-6} cells) in *C. vulgaris* cells after 72 h exposure to 0.251 mg L^{-1} AgNPs in BBM medium without glucose (-Glu) and with 0.5% glucose (+Glu). The silver content in the control cells was below the limit of quantification (< 0.1 µg 10^{-6} cells). Values are given as the mean ± SE of three biological replicates. Treatments significantly different at $P \leq 0.05$ (one-way ANOVA followed by a Newman–Keuls post hoc test) are indicated with different letters.

	Control (-Glu)	AgNPs (-Glu)	Control (+Glu)	AgNPs (+Glu)
Silver content (µg $g^{-1}_{\text{dry mass}}$)	0.00 ± 0.00^{a}	1234.10 ± 285.48^{c}	0.00 ± 0.00^{a}	404.31 ± 60.46^{b}

ROS formation

Use of the DHE probe revealed no significant difference in ROS content after either treatment with AgNPs than in the respective controls (Fig. 2A). However, treatment with AgNP (-Glu) resulted in a significant increase in ROS formation compared to the AgNP treatments with glucose (AgNP (+Glu)) (Fig. 2A). A similar pattern was observed with the H_2DCFDA probe, where exposure to AgNP (-Glu) resulted in a significantly increased value compared to all other treatments (Fig. 2B). The overall impression is that the addition of glucose reduces AgNP- induced ROS formation.

As for H_2O_2 content, exposure to AgNP (-Glu) also resulted in a significantly greater increase than all other treatments (Fig. 2C). An increase in H_2O_2 content was also observed with AgNP (+Glu) compared to the corresponding control, although it was not statistically significant (Fig. 2C). When comparing the two treatment groups, H_2O_2 formation increased after exposure to AgNP (+Glu) or AgNP (-Glu) compared to the corresponding controls, but the amount of H_2O_2 increased significantly more after treatment with AgNP (-Glu), again indicating the alleviation effects of glucose.

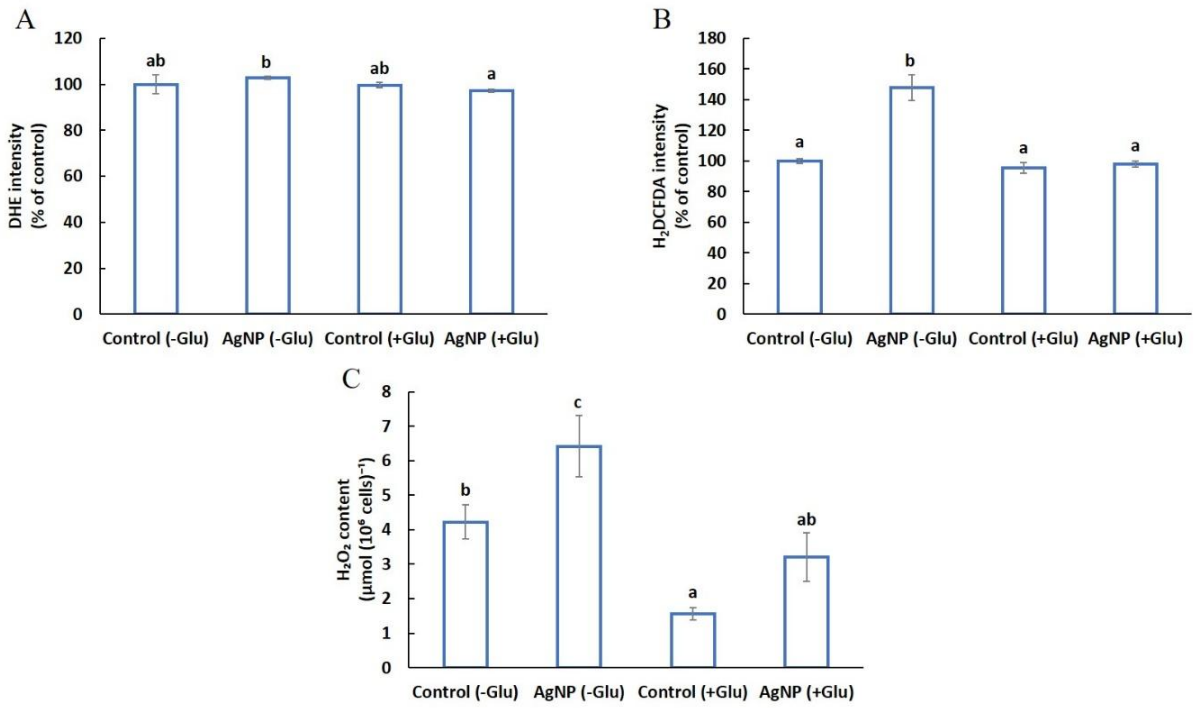

Fig. 2. The levels of reactive oxygen species (ROS) in *C. vulgaris* cells determined *in situ* after 72 h exposure to 0.251 mg L^{-1} AgNPs in BBM medium without glucose (-Glu) and with 0.5% glucose (+Glu), using dihydroethidium (DHE) (A) and 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) (B), while hydrogen peroxide (H_2O_2) content (C) was analysed in cell extracts. Values are given as the mean ± SE of six biological replicates. Treatments significantly different at $P \leq 0.05$ (one-way ANOVA followed by a Newman–Keuls post hoc test) are indicated with different letters.

Damage of membrane lipids

Changes in MDA content following AgNP treatment are presented in Fig. 3. Exposure to AgNP (-Glu) resulted in a significant 260% increase in MDA levels compared to the corresponding control (-Glu), indicating enhanced lipid peroxidation. In contrast, treatment with AgNP (+Glu) resulted in levels similar to those in the (+Glu) control, suggesting that glucose attenuates AgNP-induced damage of membrane lipids.

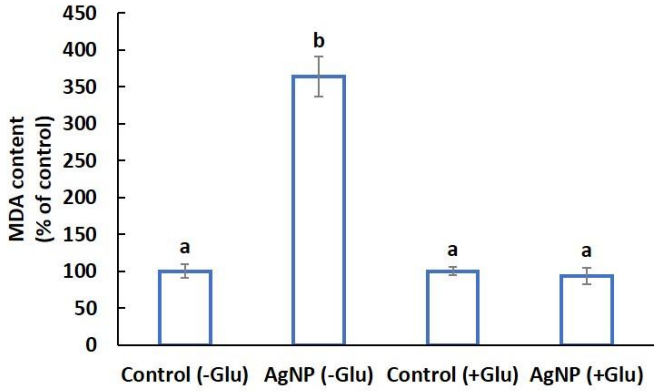

Fig. 3. Malondialdehyde (MDA) content in *C. vulgaris* cells determined after 72 h exposure to 0.251 mg L^{-1} AgNPs in BBM medium without glucose (-Glu) and with 0.5% glucose (+Glu). Values are given as the mean ± SE of six biological replicates. Treatments significantly different at $P \leq 0.05$ (one-way ANOVA followed by a Newman–Keuls post hoc test) are indicated with different letters.

Regulation of antioxidant enzyme activity

The activity of all tested antioxidant enzymes was clearly and significantly the highest after exposure to AgNPs with the addition of glucose (AgNP (+Glu)) (Fig. 4). CAT and APX showed a similar response, as no significant changes in their activities were observed with any of the other treatments (Fig. 4A, B). The greatest differences between treatments were observed for the PPX enzyme, which also showed increased activity after exposure to AgNPs without the addition of glucose (AgNP (-Glu)) compared to the corresponding control, although this increase was significantly lower in comparison to the AgNP (+Glu) treatment (Fig. 4C). The results obtained indicate that the addition of glucose has a stimulatory effect on the enzymatic antioxidant system.

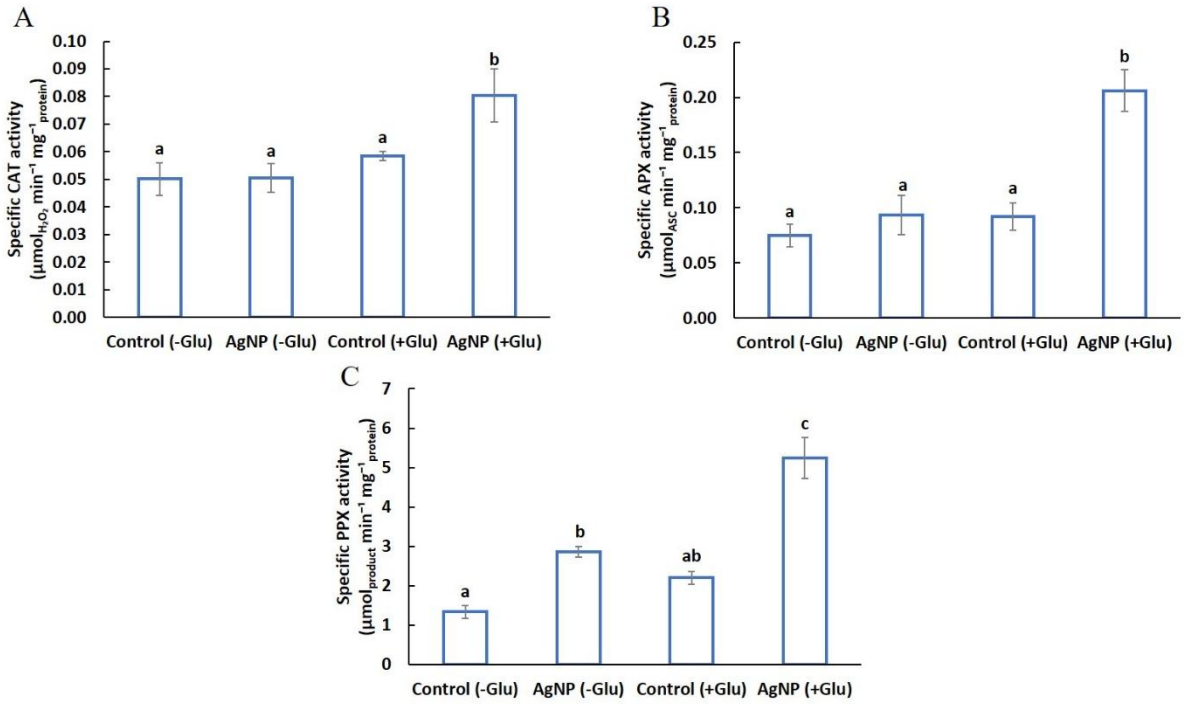

Fig. 4. Activities of the antioxidant enzymes catalase (CAT) (A), ascorbate peroxidase (APX) (B) and pyrogallol peroxidase (PPX) (C) in *C. vulgaris* cells after 72 h exposure to 0.251 mg L^{-1} AgNPs in BBM medium without glucose (-Glu) and with 0.5% glucose (+Glu). Values are given as the mean ± SE of six biological replicates. Treatments significantly different at $P \leq 0.05$ (one-way ANOVA followed by a Newman–Keuls post hoc test) are indicated with different letters.

Photosynthesis rate

As shown in Fig. 5, none of the treatments caused statistically significant changes in the rate of photosynthesis compared to their respective controls. However, a slight decrease was observed in the AgNP (-Glu) treatment relative to both its control and the AgNP (+Glu) treatment.

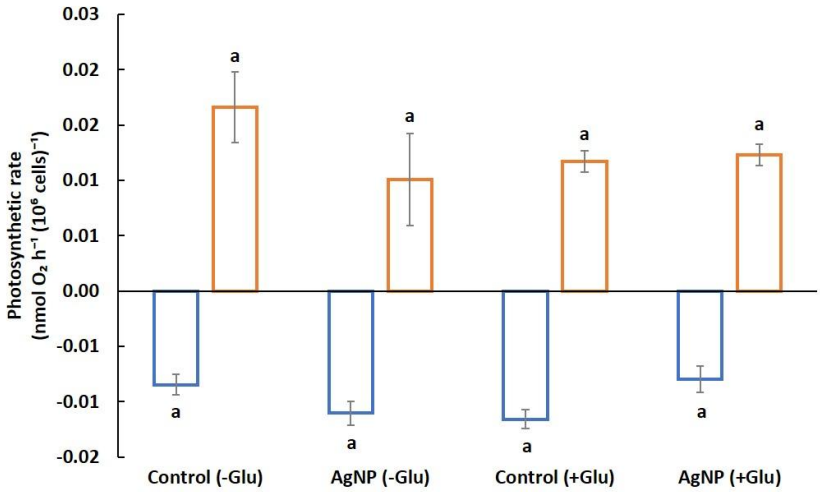

Fig. 5. Photosynthetic rate in *C. vulgaris* cells after 72 h exposure to 0.251 mg L^{-1} AgNPs in BBM medium without glucose (-Glu) and with 0.5% glucose (+Glu) in the dark (blue) and at a light intensity of 80 $\mu mol_{photons}$ m^{-2} s^{-1} (orange). Values are given as the mean ± SE of three biological replicates. Treatments significantly different at $P \leq 0.05$ (one-way ANOVA followed by a Newman–Keuls post hoc test) are indicated with different letters.

Chlorophyll *a* fluorescence parameters

The F_v/F_m decreased significantly after treatment with AgNP (-Glu) compared to the control (-Glu) as well as to AgNP (+Glu) and the control (+Glu) (Fig. 6A). A similar trend was observed in the PI_{abs}, which also showed a significant decrease after exposure to AgNP (-Glu) compared to all other treatments (Fig. 6B). No significant differences in F_v/F_m or PI_{abs} were observed between AgNP (+Glu) and its control, while the PI_{abs} value was higher in the control (-Glu) than in the control (+Glu). Interestingly, however, both F_v/F_m or PI_{abs} were significantly higher in glucose treatments compared to AgNP (-Glu), suggesting that the addition of glucose restores the effectiveness of photosynthesis.

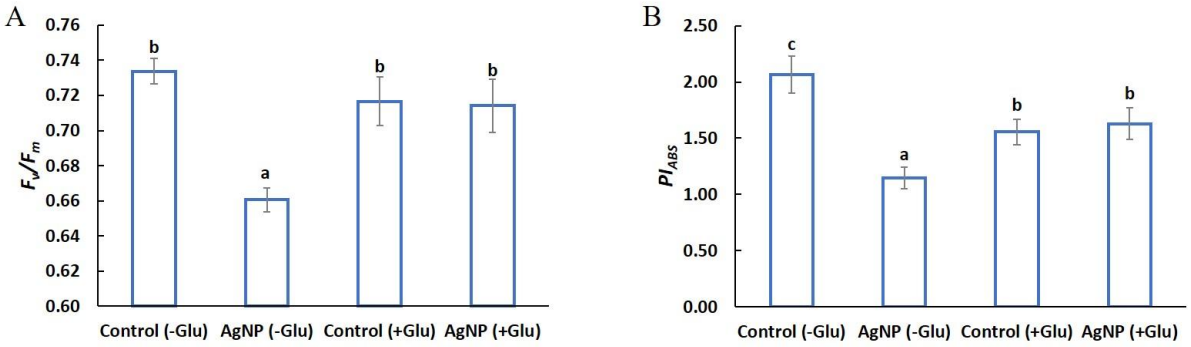

Fig. 6. Maximum quantum yield of PSII (F_v/F_m) (A) and photosynthetic efficiency index (PI_{abs}) (B) in *C. vulgaris* cells after 72 h exposure to 0.251 mg L^{-1} AgNPs in BBM medium without glucose (-Glu) and with 0.5% glucose (+Glu). Values are given as the mean ± SE of six biological replicates. Treatments significantly different at $P \leq 0.05$ (one-way ANOVA followed by a Newman–Keuls post hoc test) are indicated with different letters.

Photosynthetic pigment content

The analysis of the photosynthetic pigments showed treatment-specific responses (Fig. 7). A significant increase in Chl *a* content was observed after exposure to AgNP (-Glu) compared to the corresponding control (Fig. 7A), while no significant changes in Chl *b* or

carotenoid content were observed (Fig. 7B, C). The treatment with AgNP (+Glu) had no significant effect on the content of photosynthetic pigments compared to its control, although the addition of glucose significantly reduced the content of Chl *a* compared to AgNP (-Glu). It is also interesting to note that the treatments without glucose resulted in a significantly higher content of carotenoids than treatments with glucose addition.

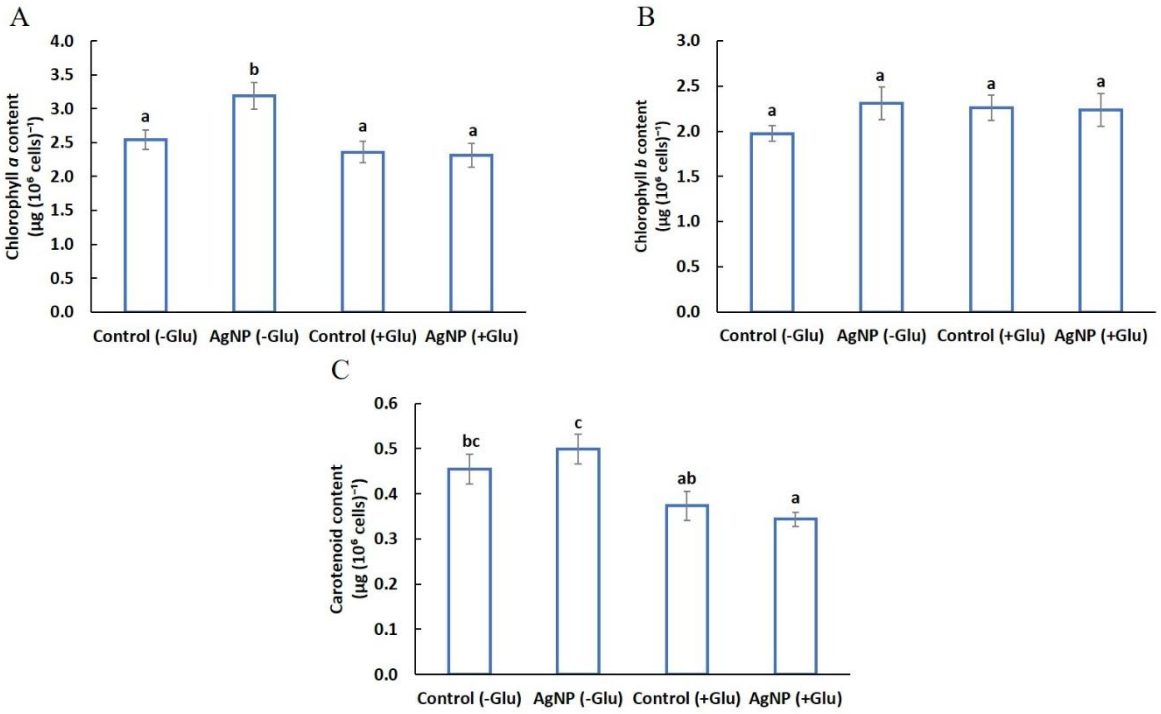

Fig. 7. Chlorophyll *a* (A), chlorophyll *b* (B) and carotenoid (C) content in *C. vulgaris* cells after 72 h exposure to 0.251 mg L^{-1} AgNPs in BBM medium without glucose (-Glu) and with 0.5% glucose (+Glu). Values are given as the mean ± SE of six biological replicates. Treatments significantly different at $P \leq 0.05$ (one-way ANOVA followed by a Newman–Keuls post hoc test) are indicated with different letters.

Ultrastructural changes

Ultrastructural changes in *C. vulgaris* cells following AgNP exposure were observed by TEM and are presented in Fig. 8. Both AgNP treatments caused visible alterations compared to their respective controls. In cells treated with AgNP (-Glu), plasmolysis, cell wall instability, and increased starch granule accumulation were evident (Fig. 8B). Addition of glucose led to enhanced formation of lipid droplets and starch granules even in the control group (Control (+Glu)) (Fig. 8C), while the most notable accumulation of starch granules was observed in cells treated with AgNP (+Glu) (Fig. 8D). These results clearly indicate that glucose addition stimulates starch production.

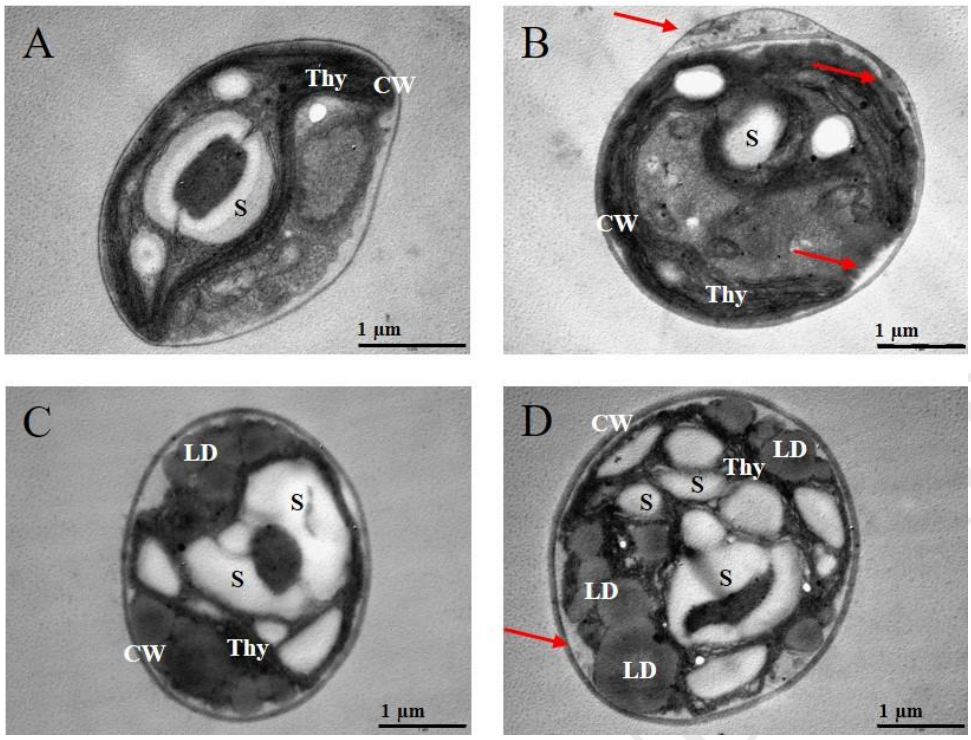

Fig. 8. Ultrastructure of control *C. vulgaris* cells in BBM medium without glucose (-Glu) (A) and with 0.5% glucose (+Glu) (C), and cells after 72 h exposure to 0.251 mg L^{-1}AgNPs in BBM medium without glucose (-Glu) (B) and with 0.5% glucose (+Glu) (D), imaged by transmission electron microscopy. Thy - thylakoids; S - starch; CW - cell wall; red arrows indicate changes in the cell wall. Bar = 1 µm.

Discussion

The increasing use of AgNPs increases their concentration in aquatic ecosystems and consequently has a negative impact on the organisms living there. According to studies, the concentration of AgNPs in wastewater treatment plant effluents worldwide ranges between 16.4 and 17 000 ng L^{-1}, while concentrations in surface waters are between 0.088 and 10 000 ng L^{-1} (Maurer-Jones et al. 2013). In our study, we chose the AgNP concentration that allows the survival of 60% of algal cells (EC_{40}) after 72 h of treatment. Given the measured and estimated concentrations of AgNPs in the environment, this concentration is environmentally relevant, as it is likely that the AgNP concentrations at micro-locations will increase to the indicated level. Furthermore, AgNPs in the environment have chronic, longer-term effects on organisms, whereas our experiment aimed to assess the acute effects following a 72 h exposure of algae to AgNPs and thus focus on the immediate toxic responses of the algae. Although microalgae are naturally photosynthetic, many species can adopt mixotrophic or heterotrophic growth in the presence of organic compounds (Castillo et al. 2021). In this study, we used glucose to induce mixotrophic growth of *C. vulgaris* and to investigate its potential mitigating effect on the toxicity of AgNPs.

Most studies that have investigated the phytotoxicity of AgNPs to freshwater algae have focused on changes in cell morphology and photosynthesis as well as silver accumulation in algal cells (Afkar, 2010; Dash et al. 2012; Mariano et al. 2020; Romero et al. 2020). In recent years, however, research has increasingly focused on oxidative stress caused by ROS and thus on the antioxidant response (He et al. 2012; Lekamge et al. 2020). In algae, ROS are produced in chloroplasts, peroxisomes, cytosol and mitochondria as by-products of photosynthesis and cellular respiration, but their accumulation is enhanced under various environmental stress

conditions, including exposure to AgNPs (Rezayian et al. 2019; Romero et al. 2020). To determine the effect of glucose on ROS formation and oxidative stress after exposure of *C. vulgaris* to AgNPs, we designed an experiment in which the algal cells were exposed to the same concentration of AgNPs in a medium without glucose and with glucose, and the results obtained were compared with each other and with the respective controls. Two indicators were used for ROS determination (Ng and Ooi, 2021), one for the total ROS content and the other for the content of $O_2^{\bullet -}$, and increased values were recorded after exposure to AgNPs in a medium without glucose (-Glu). These results are consistent with the findings obtained in previous studies when different microalgae species were exposed to AgNPs (Hazeem et al. 2019; Shanab et al. 2019; Lekamge et al. 2020; Zhao et al. 2021). However, the addition of glucose in the treatment with AgNPs in our study resulted in a significant decrease in ROS content with both indicators compared to AgNP (-Glu), suggesting that algae with an additional food source can successfully maintain the antioxidant system in the fight against ROS. This attenuating effect of glucose on the formation of ROS molecules was even more pronounced in the H_2O_2 content, which was significantly lower in the treatments with glucose, both in the control (+Glu) and AgNP (+Glu), compared to the control and AgNP treatment without glucose. This is also consistent with the results of our previous study (Komazec et al. 2023) and shows once again that algae can use an additional energy source for an effective defence against oxidative stress.

One of the harmful effects of ROS is the peroxidation of cell membrane lipids, which usually leads to the formation of MDA, a cytotoxic product that can cause irreversible damage to proteins and DNA molecules (Vavilin et al. 1998; Rezayian et al. 2019). Previous studies on different algal species have shown that increased ROS content in algal cells can lead to higher levels of lipid peroxidation upon exposure to AgNPs (Oukarroum et al. 2012; Shanab et al. 2019; Zhao et al. 2021). This was confirmed in this study, as exposure of *C. vulgaris* to AgNP (-Glu) led to a significantly increased MDA content compared to the control (-Glu). On the other hand, the addition of glucose to the culture medium in exposure to AgNPs reduced MDA production to the control level, again indicating a possible mitigating effect of glucose.

In response to the induction of oxidative stress by AgNPs, algae have a wide range of defence strategies, including the activation of enzymatic antioxidant system, to eliminate excess ROS (Romero et al. 2020). In this study, we investigated the activities of three antioxidant enzymes, all of which degrade H_2O_2 and thus protect cells from oxidative stress (Kalehjahi et al. 2023). Treatment with AgNP (-Glu) had no effect on the specific activity of CAT and APX compared to the control (-Glu). A similar result was reported by Lekamge et al. (2020), who found increased CAT activity only when the alga *Raphidocelis subcapitata* was exposed to higher AgNP concentrations (0.8 mg L^{-1}), while no significant change occurred at lower concentrations (< 0.2 mg L^{-1}), which was similar to that used in our study. In addition, studies on the effects of AgNPs on the algae *Nannochloropsis oculata* (Fazelian et al. 2020) and *Chlamydomonas reinhardtii* (Zhao et al. 2021) showed that APX activity also increased only at higher AgNP concentrations (< 5 mg L^{-1}). However, the lack of increased CAT or APX activity could also result from the altered gene regulation of these enzymes (Gupta et al. 2018), which could be a consequence of DNA damage (Cameron et al. 2018), or from binding of the enzyme to the AgNP surface, resulting in damage to the heme unit within the enzyme and consequently inhibition of its activity (Liu et al. 2020b). The latter is the more likely scenario, as exposure to AgNPs significantly increased ROS production, especially H_2O_2, and lipid peroxidation, which is undoubtedly associated with oxidative stress. The addition of glucose in the control treatment had no significant effect on CAT and APX activity, but exposure to AgNP (+Glu) resulted in significantly increased levels, suggesting that the antioxidant defence of *C. vulgaris* is more effective in the presence of glucose,

probably due to the greater availability of energy. As for the PPX, its activity increased significantly after treatment with AgNP (-Glu) compared to the control (-Glu), which is due to increased ROS and lipid peroxidation levels, as previously reported for *Chlamydomonas reinhardtii* (Zhao et al. 2021) and *Dunaliella salina* (Bahador et al. 2019). This result also suggests that PPX may be more resistant to the toxic effects of AgNPs than CAT and APX. Furthermore, the addition of glucose increased the activity of the PPX enzyme in general as a slightly, although not significantly, increased activity was also measured in the control (+Glu) compared to the control (-Glu). However, the most significant increase was recorded after exposure to AgNP (+Glu). Since all enzymes use H_2O_2 as a substrate, the increase in their activities upon treatment to AgNP (+Glu) can be related to the significantly reduced H_2O_2 content measured in the presence of glucose in the culture medium, which enables a more efficient detoxification of peroxide. From the results obtained, it can be concluded that the addition of glucose to the culture medium increases the antioxidant capacity of the algal cells, as evidenced by the increased activity of the enzymes CAT, APX and PPX, which ultimately leads to a more effective reduction in oxidative stress, e.g. by reducing ROS and lipid peroxidation levels, suggesting that protection against oxidative stress is more stable and effective in the presence of glucose due to the increased energy status of the cell.

Aquatic ecosystems are vitally important for photosynthesis, as microalgae and cyanobacteria together produce about half of the Earth's atmospheric oxygen. Consequently, any reduction in their photosynthetic activity could pose a serious ecological threat. In this study, treatment with AgNP (-Glu) resulted in a slight decrease in photosynthetic rate and a significant reduction in chlorophyll fluorescence parameters (F_v/F_m and PI_{abs}) compared to the control (-Glu), which is consistent with the results of previous studies in *C. vulgaris* (Romero et al. 2020) as well as in *Euglena gracilis* (Li et al. 2015) and *Chlamydomonas reinhardtii* (Navarro et al. 2015; Salas et al. 2019; Zhan et al. 2024). The reduction in photosynthetic rate, and consequently carbon fixation capacity and chlorophyll fluorescence parameters, has been linked to the AgNP-induced negative effects, as the decrease in chlorophyll fluorescence indicates a disruption of the electron transport chain (ETC) within PSII, which consequently translates into a decrease in photosynthetic rate (Liu et al. 2020a). In a study on *Vicia faba* leaves, chlorophyll fluorescence was reduced after AgNP treatment, which is likely due to the transfer of excited energy from chlorophyll molecules to AgNP (Queiroz et al. 2016). Moreover, the structure and function of PSII can be damaged by AgNP-induced ROS, which can disrupt the normal course of electron transport (Jiang et al. 2017); the results on chlorophyll fluorescence and photosynthetic rate obtained in this study are consistent with this finding. Indeed, elevated ROS levels can trigger lipid peroxidation, which affects all cell membranes, including thylakoids (Dash et al. 2012; Mariano et al. 2020; Kalehjahi et al. 2023). On the other hand, the increased content of photosynthetic pigments, particularly Chl *a*, recorded after treatment with AgNP (-Glu) probably indicates a compensatory response of the algal cells to the reduced photochemical efficiency. This interpretation is further supported by ultrastructural observations, which revealed clear alterations in chloroplast structure, including disrupted and disorganized thylakoid membranes as well as evident plasmolysis suggesting stress-induced damage to cellular structure. These structural damages are consistent with impaired photosynthetic performance and are likely caused by AgNP-induced oxidative stress. Previous studies have also shown that AgNP can stimulate pigment synthesis in the algae *Porphyridium purpureum* (Rudi et al. 2024) and *C. vulgaris* (Hazeem et al. 2019) as well as in *Lactuca sativa* L. seedlings (Jurkow et al. 2020), *Nicotiana tabacum* and *Trigonella foenum-graecum* (Sadak, 2019). The increased content of Chl *a* and *b* can improve the absorption of light energy to compensate for the reduced energy conversion efficiency at the thylakoid membranes (Matorin et al. 2013; Liu et al. 2021). Additionally, carotenoids also function as antioxidants in algal cells, and their increased levels likely reflect an increased

oxidative stress response due to AgNP exposure, along with enhanced light energy uptake (Zuluaga et al. 2017).

In contrast, the presence of glucose in the control medium resulted in slightly reduced photosynthesis compared to the control (-Glu) in terms of F_v/F_m and PI_{abs}, which was somewhat expected as the algal cells are more dependent on photosynthesis for food production in the absence of glucose. Obviously, there is a sufficient carbon source in the medium with glucose, which is also confirmed by the increase in the number of lipid droplets and starch granules, suggesting a metabolic shift towards heterotrophy. This is consistent with findings obtained on *Chlorella sp*. and *Nannochloropsis sp*. (Cheirsilp and Torpee, 2012), where an external organic carbon source promoted intracellular storage compound synthesis. Moreover, AgNP exposure did not result in significant changes in chlorophyll fluorescence parameters, photosynthetic rate or pigment content compared to the control (+Glu), supporting the hypothesis that the addition of 0.5% glucose provides an additional carbon and energy source that helps the algal cells to mitigate the oxidative stress caused by AgNP exposure. In fact, in the presence of glucose, the algae may be less inclined to increase pigment production as they partially meet their energy and carbon requirements heterotrophically. Ultrastructural analysis further supports this interpretation, as TEM images of algae cells after AgNP (+Glu) treatment showed an even more pronounced accumulation of starch granules and lipid droplets compared to the control (+Glu), which is likely both a stress response and a consequence of extra energy and carbon availability. Furthermore, cells after AgNP (+Glu) treatment exhibited a preserved chloroplast structure and visible thylakoid membranes, in contrast to the disorganization observed in cells after AgNP (-Glu). The maintained cellular integrity and absence of pronounced plasmolysis in the AgNP (+Glu) treatment further indicate that glucose helps stabilize internal structures and alleviate AgNP toxicity by providing sufficient energy for adequate activation of antioxidant defence mechanisms, as indicated in the oxidative stress results.

Finally, if we compare the results of treatment with AgNP (-Glu) and AgNP (+Glu), a significant increase in Chl *a* fluorescence parameters is observed, accompanied by a significant decrease in the content of photosynthetic pigments, again suggesting that in the absence of glucose, the compensatory increase in chlorophyll content is likely to be a more pronounced response to compensate for the reduction in lack of photosynthetic efficiency caused by AgNPs, and that the addition of glucose may mitigate the phytotoxic effects of AgNPs on photosynthesis in algae.

Conclusion

The acute toxicity of AgNPs to *C. vulgaris* at ecologically relevant concentrations was confirmed in this study by an increase in oxidative stress and disruption of photosynthetic processes. In the absence of glucose, AgNPs caused significant accumulation of ROS, resulting in increased lipid peroxidation, ultrastructural damage in the form of plasmolysis and cell wall degradation, and decreased photosynthetic efficiency. These effects were accompanied by little or no activation of antioxidant enzymes. In contrast, glucose supplementation attenuated the negative effects of AgNPs on *C. vulgaris* by bringing ROS accumulation and lipid peroxidation to control levels through the significant activation of all analysed antioxidant enzymes. This was accompanied by a preserved photosynthetic performance and a low degree of ultrastructural damage as well as increased lipid droplet synthesis. These results suggest that an additional carbon source such as glucose likely improves the energy status of *C. vulgaris* cells and facilitates the management of oxidative stress by supporting the activation of antioxidant defence, which in turn enables modulation of physiological and biochemical responses to AgNPs. This study demonstrates that nutrient

and energy availability significantly modulates the toxicity of nanoparticles to primary producers, emphasizing the importance of the environmental context in assessing the ecological risk of nanomaterials.

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Author contribution statement

B.K., P.P.Š. - conceptualization and design of the experiments; B.K. - nanoparticle synthesis; B.K., P.P.Š. - nanoparticle characterization; B.K., K.G. - algal exposure; B.K., S.Š. - silver content analyses; B.K., K.G. - ROS, MDA and antioxidants analyses; P.P.Š. - TEM analyses; B.K. - statistical analysis; B.K., P.P.Š. - writing – original draft preparation; B.B. - writing – review and editing; P.P.Š. - supervision. All authors have read and agreed to the published version of the manuscript.

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