

# Combined biocidal action of silver nanoparticles and ions against Chlorococcales (*Scenedesmus quadricauda*, *Chlorella vulgaris*) and filamentous algae (*Klebsormidium* sp.)

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**Abstract** Despite the extensive research, the mechanism of the antimicrobial and biocidal performance of silver nanoparticles has not been unequivocally elucidated yet. Our study was aimed at the investigation of the ability of silver nanoparticles to suppress the growth of three types of algae colonizing the wetted surfaces or submerged objects and the mechanism of their action. Silver nanoparticles exhibited a substantial toxicity towards Chlorococcales *Scenedesmus quadricauda*, *Chlorella vulgaris*, and filamentous algae *Klebsormidium* sp., which correlated with their particle size. The particles had very good stability against agglomeration even in the presence of multivalent cations. The concentration of silver ions in equilibrium with nanoparticles markedly depended on the particle size, achieving about 6 % and as low as about 0.1 % or even less for the particles 5 nm in size and for larger ones (40–70 nm), respectively. Even very limited proportion of small particles together with larger ones could substantially increase concentration of Ag ions in solution. The highest toxicity was found for the 5-nm-sized particles, being the

smallest ones in this study. Their toxicity was even higher than that of silver ions at the same silver concentration. When compared as a function of the Ag<sup>+</sup> concentration in equilibrium with 5-nm particles, the toxicity of ions was at least 17 times higher than that obtained by dissolving silver nitrite (if not taking into account the effect of nanoparticles themselves). The mechanism of the toxicity of silver nanoparticles was found complex with an important role played by the adsorption of silver nanoparticles and the ions released from the particles on the cell surface. This mechanism could be described as some sort of synergy between nanoparticles and ions. While our study clearly showed the presence of this synergy, its detailed explanation is experimentally highly demanding, requiring a close cooperation between materials scientists, physical chemists, and biologists.

**Keywords** Silver nanoparticles · Silver ions · Concentration of silver ions in equilibrium with silver nanoparticles · Algae · Toxicity · Uptake of silver by algae

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## Introduction

Owing to their antimicrobial and biocidal ability, silver nanoparticles have been used in a broad range of industrial products for more than 100 years (Nowack et al. 2011). Despite the extensive research, the mechanism of their toxicity has not been unequivocally elucidated yet. There are two important aspects which should be taken into account. First, the understanding of the toxicity of the silver nanoparticles is complicated by the coexistence of the particle and ionic forms, which are expected to perform differently either in an independent or synergistic manner (Liu and Hurt 2010). There are contradicting studies claiming either silver ions or nanoparticles as the main source of toxicity (Sotiriou and Pratsinis 2010).

Second, the toxicity of silver nanoparticles markedly depends on the targeted organism, such as the most commonly tested bacteria, further algae, and fungi.

Silver nanoparticles are highly sensitive to oxygen resulting in the formation of partially oxidized surfaces with chemisorbed  $\text{Ag}^+$  (Henglein 1998). Ion release from silver nanoparticles was shown due to a cooperative oxidation involving protons and dissolved oxygen. Further, the existence of three distinct forms of silver in a nanoparticle colloid was clearly demonstrated, namely the silver nanoparticles, dissolved silver ions, and adsorbed ions on the surface of nanoparticles (Liu and Hurt 2010). The silver ion release can be suppressed by stripping off dissolved oxygen, by the addition of reducing matter and stabilizer, by the reduction in temperature, and by an increase in pH (Liu and Hurt 2010).

Concerning the toxicity, Navarro et al. (2008a, b) showed that based on the total silver concentration, the toxicity of silver ions towards algae was 18 times higher than that of nanoparticles. When compared as a function of the concentration of the free  $\text{Ag}^+$  ions in a AgNP solution excluding the Ag bound to NPs, however, the toxicity of nanoparticles appears much higher than that of ions. For nanoparticles ranging in size from 10 to 200 nm, only 1 % of silver was in the form of ions (Navarro et al. 2008a, b). Similarly, according to Miao et al. (2009), the toxicity of silver nanoparticles was primarily due to dissolved ions. Lok et al. (2007) found that silver nanoparticles with partially oxidized surface have antibacterial activity but zero-valent nanoparticles do not. The level of chemisorbed  $\text{Ag}^+$  on the particle surface correlates with the observed antibacterial activity (Lok et al. 2007).

On the other hand, Fabrega et al. (2009) revealed that there is a specific nanoparticle effect, which cannot be explained by the effect of dissolved silver ions. Specifically, the contact between the silver nanoparticles and bacteria surface was the dominant driver of toxicity. In addition, several other studies claimed that the toxicity of silver is not exclusively due to the ions but rather to silver nanoparticles (Fabrega et al. 2009; Li et al. 2015; Kaba and Egorova 2015; Stensberg et al. 2014). Silver nanoparticles 1–10 nm in size bind preferentially to cell membranes and are incorporated into bacteria whereas larger particles are not. Nanoparticles were shown causing pitting on bacterial cell membranes leading to increased permeability and cell death (Fabrega et al. 2009).

Recently, these contradictory results were addressed by Sotiriou and Pratsinis (2010) who tested the performance of well-defined silver nanoparticles immobilized on inert  $\text{SiO}_2$  support against the bacteria *Escherichia coli*. According to these authors, the mechanism of the antibacterial activity of silver nanoparticles depends on their size. As very small nanoparticles release a substantial proportion of ions, the bactericidal activity is dominated by  $\text{Ag}^+$  rather than by nanoparticles themselves. With particles larger than ca 10 nm, the release of silver ions is substantially suppressed and the particles

themselves influence the bactericidal activity (Sotiriou and Pratsinis 2010).

To date, the primary target organisms used in studies to assess the toxicity of nanoparticles were bacteria (Panacek et al. 2006; Liong et al. 2009; Sondi and Salopek-Sondi 2004; Kvitek et al. 2008; Morones et al. 2005). Besides bacteria, higher organisms were also included; however, the published studies are much scarcer. For example, the toxicity of silver nanoparticles was tested on fungi (Navarro et al. 2008a, b; Prucek et al. 2011), algae and other fresh water species (Oukarroum et al. 2012a, b; Dewez and Oukarroum 2012; Rodriguez-Gonzales et al. 2010; Narayanan and Sakthivel 2011; Kennedy et al. 2010; Zhao and Wang 2011, 2010; Gao et al. 2009; Blinova et al. 2013; Ulm et al. 2015), fish (Bilberg et al. 2010; Scown et al. 2010), plants (Kumari et al. 2009), and terrestrial organism (Panacek et al. 2011; Roh et al. 2009).

As the aim of our study was to investigate the mechanism and ability of silver nanoparticles to suppress the growth of organisms colonizing the wetted surfaces or submerged objects, three types of algae were chosen as suitable test organisms, namely *Scenedesmus quadricauda*, *Chlorella vulgaris*, and *Klebsormidium sp.* In order to obtain reliable data on the toxicity of silver nanoparticles, well-defined silver colloids with a narrow particle size distribution and limited agglomeration are needed, whose surface properties are characterized in detail. Of equal importance are the selection of suitable organisms and the reliability of toxicity tests carried out. Therefore, our study aimed at the preparation of nanoparticle dispersions with a well-defined particle size and the detailed characterization of their surface properties, especially with respect to the silver ion release, the enhancement of the stability of silver nanoparticles in aqueous electrolyte solutions, and the nanoparticle uptake by the algae. The toxicity of the well-characterized silver nanoparticles towards algae was determined and compared with that of silver ions.

## Materials and methods

### Materials

Materials used include silver nitrate (99.9 %, Lach-Ner, Neratovice, Czech Republic), ammonia (25 % w/w aqueous solution, Lach-Ner, Neratovice, Czech Republic), sodium hydroxide (p.a., Lach-Ner, Neratovice, Czech Republic), lactose monohydrate (p.a., Lach-Ner, Neratovice, Czech Republic), D-(+)-maltose monohydrate (p.a., Lach-Ner, Neratovice, Czech Republic), D-(+)-galactose (p.a., Sigma-Aldrich, Missouri, USA), D-(+)-glucose monohydrate (p.a., Lach-Ner, Neratovice, Czech Republic), and sodium borohydride (98 %, Alfa Aesar, Karlsruhe, Germany). Polyacrylic acid sodium salt (p.a., Sigma-Aldrich, Missouri, USA) was used

to modify the surface of the silver nanoparticles. To obtain the calibration curve of free silver ions, silver chloride, bromide, iodide, and thiocyanate (all Alfa Aesar, Karlsruhe, Germany) were used. Knopp medium was prepared by dissolving potassium nitrate (p.a., Lach-Ner, Neratovice, Czech Republic), potassium hydrogen phosphate (p.a., Lach-Ner, Neratovice, Czech Republic), magnesium sulfate heptahydrate (p.a., Lach-Ner, Neratovice, Czech Republic), and iron chloride anhydrous (p.a., Lach-Ner, Neratovice, Czech Republic) in deionized water (Millipore, Praha, Czech Republic).

### Preparation of silver nanoparticles

Silver nanoparticles were prepared by two methods. In the first method, the complex  $[\text{Ag}(\text{NH}_3)_2]$  cations were reduced by monosaccharides (glucose, galactose) and disaccharides (lactose, maltose), respectively. Alternatively, the  $[\text{Ag}(\text{NH}_3)_2]$  cations were reduced by sodium borohydride, and polyacrylic acid sodium salt was used as a surfactant. The initial concentrations of the reagents in the reaction system were as follows: 1 mM  $\text{AgNO}_3$ , 5 mM ammonia, and 10 mM reducing agent. Sodium hydroxide was added to initiate the reduction. All the solutions were prepared using deionized water (Millipore). All the experiments were carried out at a laboratory temperature.

### Characterization methods

The size distribution of silver nanoparticles was determined using dynamic light scattering (DLS, Malvern, Zeta Plus) and transmission electron microscopy (EM201, Philips). Zeta potential of the nanoparticles was measured using Zeta Plus apparatus (Malvern). The total concentration of silver in the colloidal solutions was obtained using atomic absorption spectroscopy (Spectr AA880, Varian) with flame atomization technique, type of the flame A-A, wavelength 328.1 nm. The samples were diluted 10 to 100 times and acidified with nitric acid.

The optical properties of nanoparticle sols were measured using UV/visible absorption spectrophotometry (Perkin Elmer, Lambda 19) in the range of wavelengths from 300 to 800 nm. The surface plasmon resonance occurred typically at a wavelength maximum of 390 to 450 nm. WTW inoLab pH 7310 with silver ion-selective electrode and saturated calomel electrode was used for the determination of the concentration of silver ions in colloidal solution of silver nanoparticles with varying particle size distribution.

### Determination of the concentration of silver ions in the colloidal solution of silver

The concentration of silver ions was determined by a potentiometric method, based on measuring their electrochemical

potential against a reference of a 1 mM solution of silver nitrate. Both solutions were connected with a liquid salt bridge formed by a saturated potassium nitrate solution. The calibration curve for this measurement was based on the known solubility of silver chloride, bromide, iodide, and thiocyanate, with 0.1 and 0.01 mM solutions of silver nitrate also being included. In the calibration curve, the potentials were plotted against the equilibrium concentration of silver ions calculated from the solubility equilibrium of silver halides and thiocyanate (Table S1, Fig. S1).

### Determination of the uptake of total silver

Colloidal solution of silver (10 mg/L) was added to a known amount of algae dispersed in Knopp medium. After an equilibrium was achieved (96 h for all the systems), uptake of silver nanoparticles was determined using UV/vis spectrophotometry (Schwegmann et al. 2010) and potentiometry. The uptake was calculated from the difference in absorbance (for Ag NPs) and potentials (for Ag ions) at the beginning and at the end of the sorption experiment (after 96 h). Absorbance for the individual particle sizes was determined from the absorption intensity at the wavelength corresponding to the surface plasmon resonance (390 to 450 nm depending on the particle size).

### Method of testing the toxicity

ISO 8692:2012 specifies a method for the determination of the growth inhibition of unicellular green algae by substances and mixtures contained in water or by waste water. Because of the nature of the tests carried out, ISO 8692 was modified by changing the concentration of algal inoculum (cell count of about 80,000). Higher concentrations of algal inoculum in a smaller volume of the test solution in comparison with that according to ISO 8692 ensure a more accurate calculation of the 96 h  $\text{EC}_{50}$  value. Cell density and counts were measured by fluorescence microscope equipped with a type Cyrus I counting chamber. During the test time, cell counts were determined every day.

For evaluation of the biomass production and vitality of algal suspension, the method of the determination of chlorophyll-*a* according to ISO 10 260 was applied. The concentration of chlorophyll-*a* was measured spectroscopically at wavelengths of 665 and 750 nm, at the beginning and at the end of the test (after 96 h). The initial concentration of algal inoculum and the volume of the test solution were about 80,000 cells in 25 mL (including the added volume of silver sol), respectively. Neither silver nanoparticles nor silver ions have any influence on photosynthetic pigments, and consequently, no interference in the determination of the concentration of chlorophyll-*a* occurred.

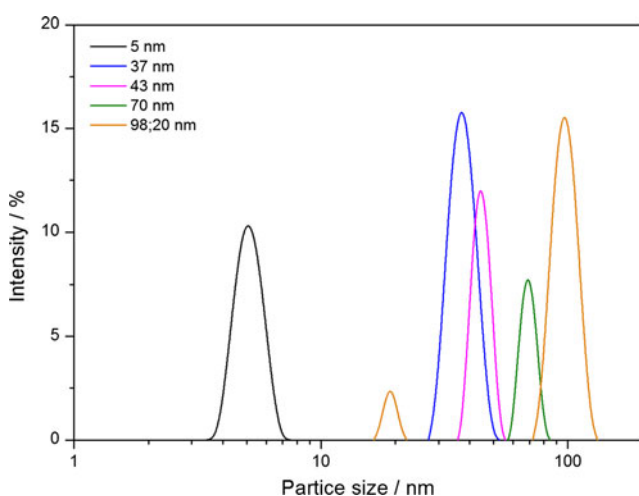
## Data analysis and statistics

The toxicity experiments were done in five replicates for all treatments. Significant differences between compared samples were determined by using Student's *t* test where *p* values were used as the threshold for statistical significance. Normality of the data and homogeneity of variance were tested using the Pearson's chi-squared test of goodness of fit and the Levene's test (Statistica software, StatSoft CR), respectively. The tests showed that the data fulfilled assumptions for the parametric statistics applied (i.e., normality of the data and homogeneity of variance).

## Results

### Physico-chemical properties of silver nanoparticles

Silver nanoparticles were prepared by two methods, namely by the modified Tollens' process (Yin et al. 2002) and by the reduction of stabilized silver salt by sodium borohydride. The average size of silver nanoparticles prepared by the modified Tollens' process was between 37 and 100 nm (Fig. 1, Table 1). The average size of nanoparticles obtained using disaccharides nanoparticles was smaller than that obtained by monosaccharides. The smallest particles only ca 5 nm in size were prepared using sodium borohydride as a reducing agent. To summarize, the strength of the reducing agent substantially influenced the size of silver nanoparticles, with monosaccharides being weaker than disaccharides and borohydride being the strongest reducing agents among those used. All prepared dispersions of silver nanoparticles exhibited very narrow size distribution, with a polydispersity value of only about 0.2.



**Fig. 1** Log-normal size distribution of silver nanoparticles prepared using various reducing agents (black-sodium borohydride; blue-maltose; magenta-lactose; green-galactose; orange-glucose) obtained by dynamic light scattering. Numbers correspond to the maximum of the particle size distribution

UV/vis absorption spectra showed the presence of a narrow surface plasmon absorption band, confirming the high dispersion of the silver nanoparticles and a low degree of their polydispersity. The plasmon band was located at the wavelength of 395–445 nm, which is in agreement with the particle size (Fig. 2). Depending on the particle size, the absorption spectrum was either blue shifted to shorter wavelengths or red shifted with the decreasing or increasing particle size, respectively. From the absorption spectra, it could be observed that the smallest particles were obtained by reducing stabilized silver salts by borohydride, which is in agreement with the data obtained by dynamic light scattering. On the other hand, the particles prepared using glucose were much larger, their size approaching 100 nm. Alternatively, the size of silver particles was determined using transmission electron microscopy. As a typical example, Fig. 3 shows silver nanoparticles prepared using sodium borohydride and maltose as the reducing agents. The average particle sizes of several samples determined from the TEM images are summarized in Table 1, and they reasonably well agree with those determined by DLS (data not shown).

An important property, which substantially influences the toxicity of silver nanoparticles, is their stability in the solution used for testing. The negatively charged silver nanoparticles, whose zeta potential equals ca  $-30$  mV, are stabilized against coagulation by a strong double-layer repulsion. For a number of colloidal systems, the critical coagulation concentration was found to vary with the inverse sixth power of the valence of the electrolyte counterions (i.e., cations for the negatively charged silver particles), according to the Schulz-Hardy rule (Israelachvili 2011). As the cultivation solution (Knopp medium) contains considerable concentration of cations, including monovalent (such as  $K^+$  and  $Na^+$ ) and multivalent (such as  $Fe^{3+}$  and  $Mg^{2+}$ ), the long-term stability of silver nanoparticles in this medium was determined. From the measurement of both the size and zeta potential, it can be concluded that the nanoparticles retained their stability even during several days' long action of the electrolyte mentioned (Fig. S2). The smallest particles prepared using sodium borohydride as the reducing agent were moreover sterically stabilized using polyacrylic acid sodium salt.

Even if the details of the mechanism of the toxicity of silver nanoparticles have not been completely elucidated yet, silver ions are known to be highly biologically active, especially in the presence of silver nanoparticles. Therefore, this study aimed to determine the concentration of silver ions in equilibrium with silver nanoparticles of varying sizes using a potentiometric technique. For the smallest particles of approximately 5 nm in size, about 6.0 % of silver was present in the form of ions (Table 1). For larger particles, this percentage was smaller with only about 0.15 % or even less of Ag being

**Table 1** An overview of silver nanoparticles and their physico-chemical properties

Reducing agent	Particle size (nm) <sup>a</sup>	Polydispersity	ζ-Potential (mV)	[Ag] (ppm) <sup>b</sup>	[Ag <sup>+</sup> ] (μM) <sup>c</sup>
Sodium borohydride	5/2	0.157	−38	78	43.1 (6.00 %)
Maltose	37/10	0.226	−34	24	0.340 (0.15 %)
Lactose	43/9	0.198	−34	24	0.220 (0.10 %)
Glucose	98/27; 20/2	0.316	−28	24	0.547 (0.25 %)
Galactose	70/13	0.235	−29	24	0.020 (0.02 %)

<sup>a</sup> The first number expresses the particles size at the maximum of the size distribution. The number after the slash is the full width at half maximum (FWHM). Data were obtained using dynamic light scattering measurements

<sup>b</sup> [Ag] total concentration of silver measured by AAS

<sup>c</sup> [Ag<sup>+</sup>] concentration of free Ag<sup>+</sup> in colloidal suspension determined by potentiometry (Fig. S1, Table S1). In the brackets: the percentage of silver in the form of ions

present in ionic form. Particles prepared using glucose as the reducing agent, which exhibit a bimodal distribution of particle size, were an exception. Due to the presence of some proportion of smaller particles about 20 nm in size, the percentage of ionic silver was higher with 0.25 %. The observed increase in the concentration of silver ions with decreasing particle size can be attributed to the dramatic increase in the proportion of surface atoms with decreasing particle size, which enables their oxidation and released into the solution.

In order to assess the interaction of the different silver species with algal cells, the uptake of silver nanoparticles and ions by algae in the Knopp medium was measured. The total amount of algae used in the uptake experiment corresponded to the concentration of chlorophyll-*a* of 187 ± 30 μg/L. The concentration of silver in the Knopp medium was 1 ppm. For the smallest nanoparticles (5 nm), 0.94 and 0.06 ppm of silver was present in the form of nanoparticles and ions, respectively. While about 20 % of the smallest nanoparticles were adsorbed on the surface of Chlorococcales, the uptake of larger silver nanoparticles was much lower—about 10 and 5 % for

nanoparticles about 40 and 70 nm in size, respectively (Fig. 5a). The decrease in the concentration of the free silver ions due to the adsorption was nearly 40 % for the smallest particles and much more (from 70 to 90 %) for the larger ones (Fig. 5b).

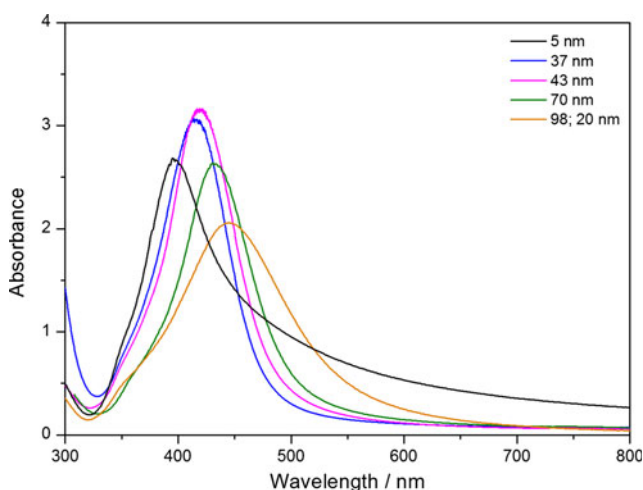
The filamentous algae *Klebsormidium sp.* tended to form poorly defined agglomerates, which influenced the sorption of silver particles on their surface. Consequently, the uptake of nanoparticles was much higher in comparison with Chlorococcales, especially for the smallest nanoparticles.

### Toxicity

For the Chlorococcales *S. quadricauda* and *C. vulgaris*, the toxicity was quantified using 96 h EC<sub>50</sub> (according to the modified ISO standard 8692). The smallest particles used in the test (5 nm) were the most toxic with 96 h EC<sub>50</sub> values of approximately 1 mg/L (Fig. 4). The toxicity of particles 5 nm in size and that of Ag<sup>+</sup> in the form of dissolved silver nitrate was not significantly different (*p* > 0.05). For the majority of samples, the 96 h EC<sub>50</sub> values for *C. vulgaris* were less than those for *S. quadricauda*. Only for Ag<sup>+</sup> and particles of 37 nm in size 96 h EC<sub>50</sub> values were not significantly different.

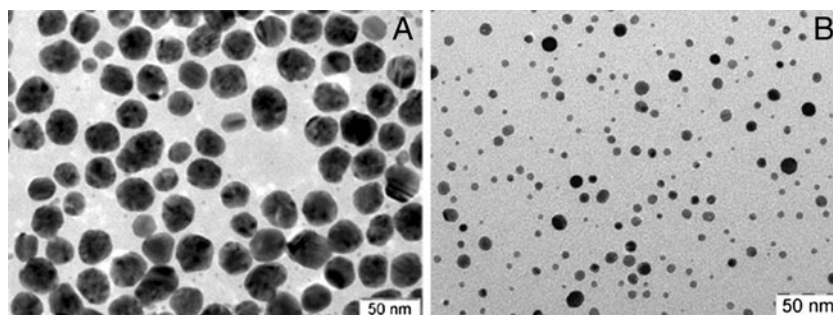
Assuming all the particles had the same density and volume, their number could be calculated by dividing the total mass of all particles by the product of the density and volume of a particle (which corresponds to the mass of a particle). As a result, the number of particles at the same total silver concentration was proportional to (dimension)<sup>−3</sup>. Consequently, for particles whose size differed by an order of magnitude (such as 5 vs. 50 nm), the number of smaller particles was by three orders of magnitude greater than that of larger ones, which also enhanced their toxicity.

For the filamentous algae *Klebsormidium sp.*, the above procedure could not be applied because of the impossibility to count the number of living and dead algae. Therefore, the toxicity test was based on the determination of the concentration of chlorophyll-*a* at the beginning and at the end of the



**Fig. 2** UV/vis absorption spectra of silver nanoparticles prepared using different reducing agents (black-sodium borohydride; blue-maltose; magenta-lactose; green-galactose; orange-glucose). Numbers correspond to the maximum of the particle size distribution

**Fig. 3** TEM images of silver nanoparticles prepared via reduction by maltose (a) and sodium borohydride (b)



experiment (after 96 h). This method was also used for Chlorococcales for the data to be comparable (Fig. 6). For Chlorococcales, the effect of particle size was more pronounced than for filamentous algae. Generally, for all three algae, the toxicity of silver ions and the smallest particles was high even at the concentration as low as 1 ppm. The toxicity of  $\text{Ag}^+$  was higher than that for 5-nm particles ( $p < 0.05$ ). The difference in toxicity for the remaining two algae was not statistically significant ( $p > 0.05$ ). The toxicity of larger particles depended on the algae species. For *Klebsormidium sp.*, the toxicity of particles 40–70 nm in size was not significantly different ( $p > 0.05$ ), being considerable already at the concentration of 3 ppm (a decrease in the chlorophyll-*a* concentration of about 80–90 %), which was significantly greater than for Chlorococcales ( $p < 0.05$ ). From the comparison of both Chlorococcales, it could be observed that *C. vulgaris* was significantly more vulnerable than *S. quadricauda*. While at the silver concentration of 10 ppm for *C. vulgaris*, the decrease in chlorophyll-*a* concentration was almost 100 %, and for *S. quadricauda*, the decrease in chlorophyll-*a* was significantly smaller, being only about

60 % for particles 40–70 nm in size (no significant differences in these range of particle size) (Fig. 6). From the comparison of the values of 96 h  $\text{EC}_{50}$  and the decrease in chlorophyll-*a* concentration (Figs. 4 and 6) for both Chlorococcales, it could be concluded that both sets of data were in reasonable agreement.

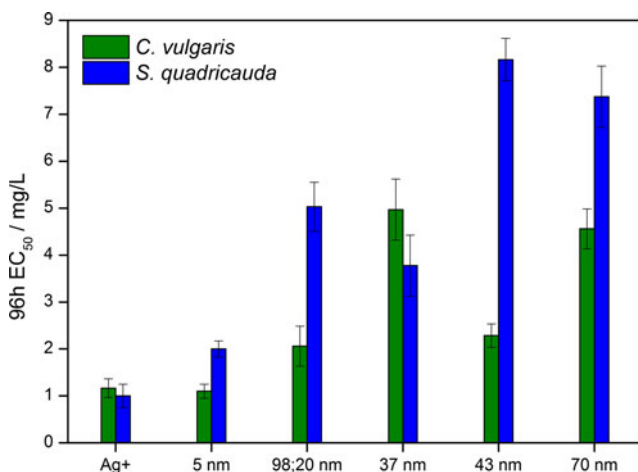
Residues of the reactants used for the preparation of silver nanoparticles did not exhibit any toxicity against the algae tested as was proved by blank experiments. This conclusion is in agreement with the data published by Suchomel et al. (2015).

## Discussion

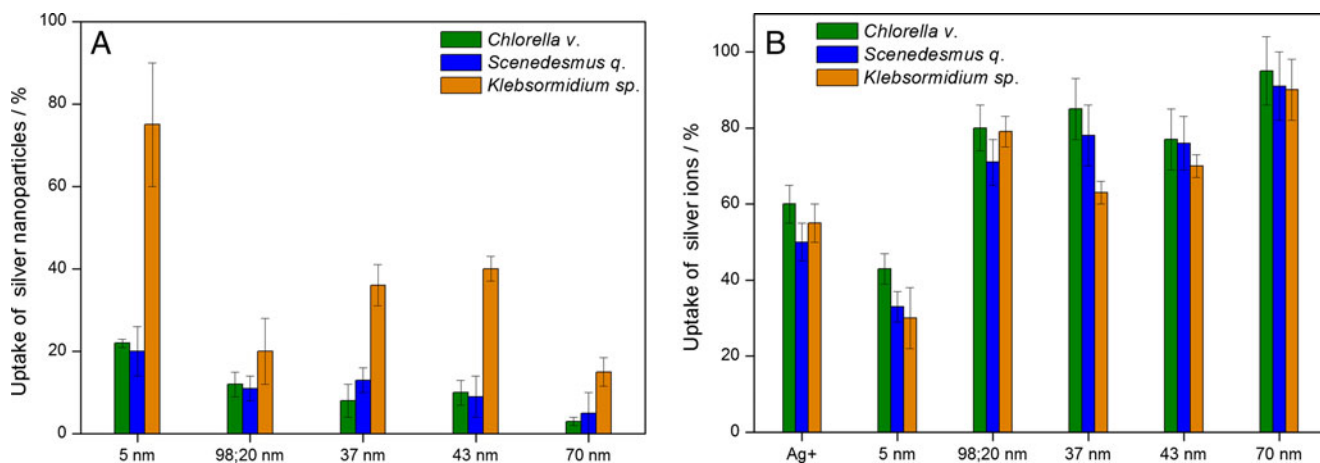
In the present study, the toxicity of silver nanoparticles and ions was investigated for Chlorococcales *S. quadricauda*, *C. vulgaris*, and filamentous algae *Klebsormidium sp.* The dispersion of silver nanoparticles consisted of primary particles as agglomeration was not detected. Zeta potential of  $-28$  to  $-36$  mV was sufficient to stabilize the nanoparticles.

From the general mechanistic ideas, it follows that several mechanisms of the toxicity of silver nanoparticles can be proposed. First, nanoparticles can puncture the cell wall or diffuse through pores within the cell wall, which can lead to the mechanical destruction of the cells or their poisoning by silver ions released from the particles inside the cells. As the diameter of pores in the cell wall of *S. quadricauda* is in a range from 5 to 20 nm, only very small nanoparticles can get inside (Bisalputra and Weier 1963). However, larger nanoparticles of approximately 50 nm in size may act as binding agents between algal cells. In this way, the formation of aggregates can inhibit the growth of algal cells (Harris 2009). Alternatively, the nanoparticles can enter the cells by endocytosis (Wang et al. 2011). From our observation, it is difficult to distinguish whether silver nanoparticles are located on the external surface of cells or inside using optical microscopy (an example is shown in Fig. S3)

Second, the toxicity can be due to the silver ions released from the nanoparticles in the aqueous solution. In this mechanism, the activity of the free silver ions can be substantially reduced due to their precipitation by reacting with anions, such as chlorides, dissolved in the solution. Our observation

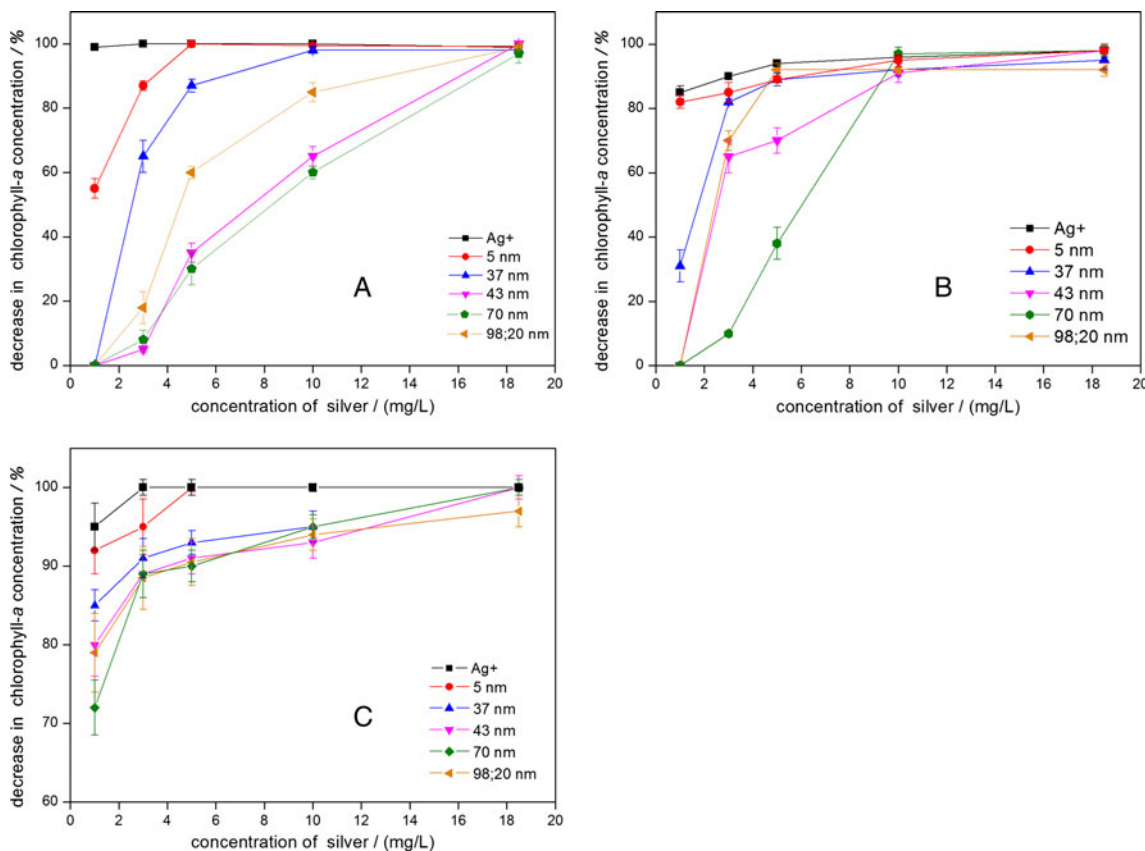


**Fig. 4** Toxicity of silver nanoparticles and ions expressed as 96-h  $\text{EC}_{50}$  for two Chlorococcales algae. Error bars are the sample standard deviation from five measurements. 96-h  $\text{EC}_{50}$  for  $\text{Ag}^+$  and 5-nm particles were not significantly different. 96 h  $\text{EC}_{50}$  values for *C. vulgaris* were significantly different from those for *S. quadricauda* for particles 5, 98:20, 43, and 70 nm in size, while those for  $\text{Ag}^+$  ions and particles 37 nm in size were not



**Fig. 5 a** Uptake of silver nanoparticles by *S. quadricauda*, *C. vulgaris*, and *Klebsormidium sp.* in the Knopp medium after 96 h determined spectrophotometrically from the difference in absorbance at the beginning and the end of sorption experiment. The total concentration of silver was 1 ppm, and the proportion of nanoparticles and ions are shown in Table 1. The concentration of chlorophyll-*a* was 187 ± 30 µg/L. Error bars are the sample standard deviations from triplicate

measurements. **b** Uptake of silver ions by *S. quadricauda*, *C. vulgaris*, and *Klebsormidium sp.* in the Knopp medium after 96 h determined potentiometrically from the difference in potentials at the beginning and the end of sorption experiment. The total concentration of silver was 1 ppm, and the proportion of nanoparticles and ions are shown in Table 1. The concentration of chlorophyll-*a* was 187 ± 30 µg/L. Error bars are the sample standard deviations from triplicate measurements



**Fig. 6** Decrease of chlorophyll-*a* content in *S. quadricauda* (a), *C. vulgaris* (b), and *Klebsormidium sp.* (c) exposed to silver nanoparticles and ions, respectively, for 24 h (consider the different scales of Y-axes). The experiments were conducted in quintuplicate, and the results are shown as the mean with standard deviations. The concentration of silver in Knopp medium was 1, 3, 5, 10, and 18.5 mg/L. Designation of samples according to the reducing agents: black-sodium borohydride, blue-maltose, magenta-lactose, green-galactose, orange-glucose. The toxicity of Ag<sup>+</sup> was

significantly different from that for 5-nm particles for *S. quadricauda* and not significantly different for *C. vulgaris* and *Klebsormidium sp.* For *Klebsormidium sp.* the toxicity of particles 40–70 nm in size was not significantly different from each other. At the concentration of 3 ppm, the toxicity of particles 40–70 nm in size was significantly different for *Klebsormidium sp.* from that for Chlorococcales. The toxicity of particles 40–70 nm in size was significantly different for *C. vulgaris* in comparison with that for *S. quadricauda*

showed that this mechanism is not probable because the concentration of silver ions in equilibrium with silver nanoparticles is very low. For particles 20–100 nm in size, it achieved only about 0.2 % of the total concentration of silver. Such a low concentration seems insufficient for the observed toxicity.

Third, the silver nanoparticles can adsorb onto the cell surface and release ions, which then can diffuse into the cell interior. A substantial adsorption of various nanoparticles on the surface of cells was already reported in several studies (Liu and Hurt 2010; Anjali et al. 2012; Ofer et al. 2004). This mechanism can be substantially enhanced by chemical action (oxidative processes) of the functional groups located on the cell surface on the silver particles leading to an enhanced release of ions. The toxicity of silver ions can be rather high as their diffusion lengths are short and the precipitation due to salts or complexation by species in the solution is much less probable in comparison to the case when silver ions are freely diffusing in the volume of the Knopp solution which contains the algal species. Ionic silver inside the cells inhibits respiratory enzymes and induces oxidative stress upon generation of reactive oxygen species (Kim et al. 2007).  $\text{Ag}^+$  may also bind to sulfur- and phosphorus-containing molecules (cystein, taurin, etc.) involved in cell antioxidant defense (Pappa et al. 2007).

Further, it was hypothesized that the contact with nanoparticles can induce the formation of new pores in the membrane through lipid peroxidation mechanisms, making it less selective. This process may be toxic to the cell, leading to inactivation of enzymes and proteins (Halliwell and Gutteridge 2007). Of course, several mechanisms can act in parallel or even can synergistically increase the toxicity. Inside the cell, the interaction of  $\text{Ag}^+$  with functional groups, such as thiols of proteins or enzymes, causes the inhibitory effect as silver ions are known to have a high affinity with thiol groups. It was reported that some metals (such as Ag or Cu) in biological systems can increase the levels of oxidative stress (Pinto et al. 2003).

Our results showed that the toxicity of very small silver nanoparticles (5 nm in size in this study) is comparable with that of ions added as a salt at the same silver concentration. However, even for such small particles, only about 6 % of silver was present as ions. Consequently, if compared as a function of the  $\text{Ag}^+$  concentration alone, the toxicity of silver ions in equilibrium with silver nanoparticles was about 17 times higher than that due to dissolved nitrate. This observation proves that the silver toxicity cannot be exclusively due to silver ions. If it were the case, then the toxicity would be directly proportional to the concentration of silver ions. Consequently, there should be some combined action of silver particles and silver ions. This combined action seems to be due to substantial uptake of silver nanoparticles by algal surface, especially for the small particles 5 nm in size. Owing to the close contact with the surface of cells, the nanoparticles

can substantially enhance the effect of freed silver ions. This common action can be described as some sort of synergy.

The difference in the response to the exposure to silver nanoparticles among various algae could be due to the differences in their cell wall structure. The determined higher toxicity of silver nanoparticles towards *C. vulgaris* in comparison with *S. quadricauda* may be due to its thinner pectic layer and less-resistant structure of the cell wall (Bisalputra and Weier 1963). It should be also taken into account that the mechanism of the toxicity of nanoparticles can be influenced by the tendency of algae towards formation of colonies. The inside of such a colony can be protected against the toxicity, which is then restricted to the peripheral part of the colony.

While our study clearly showed the presence of the synergy action between silver nanoparticles and ions, the detailed explanation of this complex mechanism is experimentally highly demanding. The necessary prerequisite for the successful studies is the availability of well-defined silver nanoparticles with a very narrow particle size distribution, which exhibit an excellent stability against agglomeration, and the precise determination of the concentration of silver ions in equilibrium with particles. Further, a broad range of organisms should be included in such comparative studies. This task requires a very close cooperation between materials scientists, physical chemists, and biologists.

## Conclusions

To sum up, the results obtained show that silver nanoparticles exhibit a substantial toxicity towards Chlorococcales and filamentous algae, which mostly correlated with their particle size. The silver nanoparticles exhibited very good stability against agglomeration even in the presence of multivalent cations. The concentration of silver ions in equilibrium with nanoparticles drastically depends on the particle size, achieving about 6 % for the particles 5 nm in size and decreasing to about 0.1 % or even less for larger particles (40–70 nm). Even relatively small proportion of small particles together with larger ones can substantially enhance the ion concentration. The uptake of nanoparticles by the cells seems to play an important role, which is substantial only for small particles in the case of Chlorococcales. The highest toxicity was found for the 5-nm-sized particles, which are the smallest ones in this study. Their toxicity was comparable with that of silver ions at the same silver concentration. The discussion of the possible mechanisms of the toxicity shows that it seems complex with an important role played by the adsorption of silver nanoparticles on the cell surface and ions released from the particles.

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