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Silver accumulation in the green microalga *Coccomyxa actinabiotos*: toxicity, *in situ* speciation and localization investigated using synchrotron XAS, XRD and TEM

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ABSTRACT

Microalgae are good candidates for toxic metal remediation biotechnologies. This study explores the cellular processes implemented by the green microalga \textit{Coccomyxa actinabiots} to take up and cope with silver over the concentration range $10^{-7}$ to $10^{-2}$ M Ag$^+$. Understanding these processes enables to assess the potential of this microalga in view to applications for bioremediation. Silver \textit{in situ} speciation and localization were investigated using X-ray Absorption Spectroscopy, X-Ray diffraction and transmission electron microscopy. Silver toxicity was evaluated by monitoring microalgal growth and photochemical parameters. Different accumulation mechanisms were brought out depending on silver concentration. At low micromolar concentration, microalgae fixed all silver initially present in solution, trapping it inside the cells into the cytosol, mainly as unreduced Ag(I) bound with molecules containing sulfur. Silver was efficiently detoxified. When concentration increased, silver spread throughout the cell, particularly entering the chloroplast where it damaged the photosystem. Most silver was reduced to Ag(0) and aggregated to form crystalline silver nanoparticles of face-centered cubic structure, with a mean size of 10 nm. Additional minor interaction of silver with molecules containing sulfur indicated the concomitant existence of the mechanism observed at low concentration and/or nanoparticle capping. Nanoparticles were observed in chloroplasts, in mitochondria, on the plasma membrane, on cytosolic membrane structures and in vacuoles. Above $10^{-4}$ M Ag$^+$, damages were irreversible and cell died. However, high silver amounts remained confined inside microalgae, showing their potential for the bioremediation of contaminated water.

KEYWORDS. Silver, microalgae, speciation, reduction, nanoparticles, synchrotron X-ray absorption spectroscopy, EXAFS, XANES, X-ray diffraction, electronic microscopy.
INTRODUCTION

Contamination of the environment by heavy metals and radionuclides is a world concern which evolves with changing human uses. Among these contaminants, silver constitutes one of the most toxic metals in aquatic environments, at the same level as cadmium and chromium, surpassed only by mercury.\(^1\) Silver toxicity depends on its speciation, the free cationic form \(\text{Ag}^+\) being highly toxic.\(^1,2\) Silver has long been used in the photographic and imaging industry, in the electrical and electronics industry, in jewelry, in the manufacture of silverware and coinage\(^3,4\) as well as for the treatment of diseases and infections.\(^5\) Recently, applications of its antimicrobial properties strongly developed, silver being now embedded in consumer products such as plasters, textiles, food containers, toothpaste or air filters in the form of nanoparticles and colloids.\(^6\) Traditionally, silver is discharged into the environment from e.g. mining and industrial applications\(^4\). But it is also discharged nowadays from the normal use of consumer products which release silver nanoparticles, colloids and \(\text{Ag}^+\).\(^7,8\) Additionally, silver also constitutes one of the main gamma emitting radioactive contaminants present in liquid effluents issuing from nuclear pressurized water reactors operating in normal conditions, representing up to 48\% of the gamma emitting radionuclides released.\(^9\)

Remediation of effluents and of environmental water contaminated by heavy metals and radionuclides is currently mostly performed using conventional physico-chemical methods such as precipitation, oxidation/reduction or adsorption on ion-exchange resins. These methods suffer from several drawbacks including cost, intolerance to organic species, little efficiency for the removal of very low contaminant concentration and generation of large secondary waste volumes.\(^10\) Biological remediation technologies based on organisms such as bacteria, fungi and plants have offered competitive alternatives in various fields. They
generally present a high efficiency, a wider field of application and lower cost and impact on
the environment than physico-chemical technologies.\textsuperscript{11,12} Microalgae are good candidates for
heavy metal and radionuclide bioremediation strategies owing to their ability to fix a wide
range of contaminants and to resist to their chemical toxicity thanks to their large surface-to-
volume ratio, the structure of their cell wall carrying functional groups able to bind and
immobilize contaminants and various mechanisms enabling metal incorporation and
subcellular sequestration, excretion, or detoxification by speciation changes.\textsuperscript{13} Algae-based
biotechnologies for pollution remediation employ common green algae such as \textit{Chlorella},
\textit{Scenedesmus}, \textit{Cladophora}, cyanobacteria or consortia of both.\textsuperscript{14} Best metal accumulation
performance is obtained with microalgal species isolated from long-term metal contaminated
sites.\textsuperscript{13} Recently, a unicellular green microalga, \textit{Coccomyxa actinabiotis}, was isolated from an
extreme environment contaminated with radioactive silver.\textsuperscript{15} High fixation capacities were
demonstrated for non-radioactive silver, namely 20 mg Ag g\textsuperscript{-1} fresh weight (FW), \textit{i.e.} about
200 mg Ag g\textsuperscript{-1} dry weight (DW), among the higher values reported for various organisms.
When exposed to high Ag\textsuperscript{+} concentrations, up to 10\textsuperscript{-2} M, most silver accumulated inside the
cell rather than on the mucilage surrounding the cell and the cell wall and formed silver
aggregates.\textsuperscript{16} \textit{C. actinabiotis} showing also an extreme radiotolerance\textsuperscript{15}, it is therefore an ideal
candidate in view to remove stable and radioactive silver both for remediation of
contaminated environmental water and for its recovery from industrial effluents.

The present study aims at exploring some of the molecular and cellular mechanisms of
silver accumulation and toxicity in \textit{C. actinabiotis} in order to establish the potential of this
promising microalga for bioremediation. The fundamental understanding of the mechanisms
of silver uptake and toxicity constitutes the essential prerequisite for any biotechnological
applications. As a result, key parameters can be controlled and optimized. Silver speciation
was investigated directly \textit{in situ} in microalgae suspended in Ag\textsuperscript{+} solutions ranging from \textmu{}M to
mM concentrations. As mentioned above, metals in excess relative to the cellular metabolic needs must be stored and/or their speciation modified to make them less toxic or facilitate their transport. Reduction processes, chelation by specific peptides or proteins such as ferritin, metallothioneins and phytochelatins or by small molecules may be involved. A variety of analytical or spectroscopic methods have been used to study the speciation of metals in biological and environmental media, such as UV-visible, infrared, Raman, X-ray Absorption Spectroscopy (XAS), laser spectroscopy, mass spectrometry, nuclear magnetic resonance or separation techniques followed by elemental analysis. XAS, comprising X-ray Absorption Near-Edge Structure (XANES) and Extended X-ray Absorption Fine Structure (EXAFS) spectra depending on the energy range above threshold, probes the near chemical environment of the central cation within 4-5 Å, with a sensitivity of up to tens of ppm. Technological advances made XAS suitable to study the speciation of metals also in situ in living organisms. In this work, XAS (XANES and EXAFS), complemented by X-Ray diffraction (XRD), were used to investigate the silver speciation in C. actinabiotis, highlighting various oxidation states, coordination, and structure depending on the silver concentration. The progressive formation and localization of silver nanoparticles was investigated at different silver concentrations using electron microscopy (TEM), thus supplementing previous localization work. Toxic effects of such Ag⁺ concentrations to C. actinabiotis were then evaluated by monitoring physiological and biochemical parameters such as microalgal growth and photosynthetic capacity which are commonly used to assess metal toxicity to microalgae. The combination of physiological, spectroscopic and physico-chemical investigations using techniques such as XAS, XRD, and TEM revealed the implementation of different cellular processes in the microalgae depending on silver concentration. Considerations about the remediation capabilities in view to use C. actinabiotis for the clean-up of contaminated water are presented.
MATERIALS AND METHODS

**Algae culture.** The microalga *Coccomyxa actinabiots* used in this study is described previously.\(^{15}\) Algal biomass was grown in batch mode in 800 mL round-bottom flasks aerated on an orbital shaker (Innova 2300, New Brunswick Scientific, Enfield, CT) at 100 rpm, under a continuous illumination of 70 \(\mu\text{E.m}^{-2}.\text{s}^{-1}\), at 21 ± 2°C, in a modified Bold Basal Medium (BBM) culture solution (Sigma-Aldrich, Saint-Louis, MO) diluted twice with deionized water, and regularly sub-cultured to maintain cell growth.

Microalgae were harvested in the growing phase by centrifugation (2000 \(g\), 5 min, 4°C), washed by three successive re-suspensions in deionized water followed by centrifugations (same conditions) to remove external elements coming from the culture medium, and finally re-suspended in ultrapure water (MilliQ, Millipore) at 2 \(g_{\text{FW}}.L^{-1}\) to perform the experiments. Algae were exposed to silver nitrate (AgNO\(_3\) Carl Roth GmbH, Karlsruhe, Germany) in ultrapure water rather than in culture medium in order to control silver speciation, which was the free form Ag\(^+\) in all experiments. The microalga *C. actinabiots* is known to grow naturally in ultra-pure water\(^{15}\) where it was discovered, so that this medium change hardly affects its metabolism during the silver exposure protocol. Particularly, the photochemical parameter \(F_v/F_m\) of control algae varied by less than 10% over the time laps of exposure in pure water (Supplementary Figure S1A).

**X-ray Absorption Spectroscopy**

**Sample preparation.** The speciation of silver accumulated by microalgae was studied *in situ* using XAS. Microalgae were exposed to 10\(^{-6}\) M, 10\(^{-5}\) M and 10\(^{-4}\) M Ag\(^+\) for 16 h, and to 10\(^{-3}\) M Ag\(^+\) for 5 h, in polyethylene terephthalate glycol-modified (PETG) flasks, and incubated in the light, temperature, and aeration conditions listed above. After exposure, algae were
harvested by centrifugation and washed three times with water (2000 g, 5 min, 4°C). Silver concentration was determined in aliquots of pellet and supernatant by ICP-MS as described below. Pellets, in the form of a thick paste, were placed in polypropylene sample holders, sealed with Kapton®, plunged into liquid nitrogen, and kept at -80°C until analysis.

**XAS measurements.** Silver K-edge XANES and EXAFS spectra were collected at the Rossendorf Beamline at the European Synchrotron Radiation Facility (ESRF, Grenoble, France). The beamline was equipped with a Si(111) double crystal monochromator, operated in channel-cut mode, and calibrated at the Ag K-edge energy of a Ag metal foil. Two Pt-coated Si mirrors were used to collimate the X-ray beam and achieve third-order harmonics rejection. Spectra were collected at 15 K using a 4He cryostat. Samples were measured in fluorescence mode using a 13-element high purity Ge detector (Canberra) and in transmission mode using ionization chambers.

**XAS analysis.** XANES spectra were calibrated in energy using the Ag foil at 25514 eV (first point in the derivative) and normalized using the Athena code. Silver form in the samples was qualitatively assessed by phenomenological comparison with reference spectra, namely Ag foil for Ag(0) metallic silver and Ag2S for Ag-S bonding with formal oxidation state Ag(I). EXAFS data treatment was performed using the Athena code after background subtraction and normalization with Ifffit routine, and then fitted using the Artemis code. The EXAFS curves were extracted and \( k^2 \)-weighted (\( k = \text{wavenumber in Å}^{-1} \)) for Fourier transformation between ca. 2.5 and 12 Å\(^{-1} \). The fitting procedure was performed in real R space between 1 and 6 Å. Phases and amplitudes were calculated using Feff84 code. For all Ag-Ag contributions, face-centered cubic (fcc) metallic silver was used as a model compound (crystallographic...
data from Kittel\textsuperscript{28}). 7 scattering paths corresponding to a cluster of 5.8 Å were selected for the adjustment procedure: at 2.89 Å (2 legs), 4.09 Å (2 legs), 5.00 Å (2 legs), 5.39 Å (3 legs), and 5.78 Å (2, 3 and 4 legs). According to the fcc structure, all the paths were linked together in the fitting procedure, so that only one Ag-Ag distance and one coordination number were refined. When Ag-S contribution was also needed in the fit, it was calculated using the structure of Ag\textsubscript{2}S (crystallographic data from Frueh\textsuperscript{29}). The spectra of samples exposed to low silver concentrations were best fitted using a combination of Ag-S and Ag-Ag single shell contributions; whereas the spectra of samples exposed to the higher silver concentrations were fitted with the previously specified seven Ag-Ag contributions together with an additional Ag-S single shell contribution.

**X-ray diffraction.** Silver speciation at high concentration was complemented by XRD measurements. Algae were exposed to 10\textsuperscript{-2} M Ag\textsuperscript{+}, in PETG flasks, for 3 h, in the light or in the dark, in the conditions described above. After exposure, algae were harvested by centrifugation and washed twice with water (2000 g, 5 min, 4°C). Pellets were frozen in liquid nitrogen and kept at -80°C. Right before analysis, samples were freeze-dried and ground into a fine powder. A drop of water was added. The obtained paste was homogeneously spread on zero-background silicon sample holders, allowed to dry, and covered with a Kapton® film. A control sample (algae not exposed to silver) was prepared in the same conditions to measure the algal baseline. Measurements were performed in Bragg-Brentano geometry, on a PANalytical X'Pert diffractometer equipped with an X'Celerator linear detector, at $\lambda = 1.54$ Å (Cu K\textsubscript{a} emission lines). International Centre for Diffraction Data (ICDD) database (http://www.icdd.com) was used to identify peaks; datasheet 00-004-0783 was used as reference for fcc silver. To determine the crystallite size, the three most intense peaks were fitted assuming a pseudo-Voigt shape function. A Scherrer type analysis$^{30}$ was performed
considering the resulting full widths at half maximum corrected from the instrumental resolution.

**Transmission electron microscopy.** Silver localization in microalgae exposed to 10^{-4} M and 10^{-2} M Ag^+ was analyzed using TEM as described in Leonardo et al. 2014. To determine the nanoparticle size distribution, the size of all particles in a given subcellular compartment or in a given section of a compartment was measured.

**Inductively-coupled plasma-mass spectrometry.** Silver uptake was assessed directly from silver concentration in microalgae using an inductively-coupled plasma-mass spectrometry (ICP-MS) apparatus described previously at m/z = 107 and 109, using calibration solutions in 1% HNO_3 (v/v). Prior to ICP-MS analysis, algae pellets were mineralized by suspension in 5 mL of concentrated HNO_3/HCl (2/1 v/v) (Suprapur® HNO_3 and HCl, Merck, Darmstadt, Germany) for 3 h at 170°C before dilution to 1% HNO_3 (v/v).

**Silver toxicity assessment.** Silver toxicity was assessed by monitoring the maximum quantum yield of photosystem II $F_v/F_m$ and the cellular growth during silver exposure and recovery. After exposure for 2 h to 10^{-7} M to 10^{-3} M Ag^+ in the conditions described above, in the light, microalgae were harvested by centrifugation, washed twice with water (2000 g, 5 min, 4°C) and allowed to recover in the culture conditions described above.
RESULTS AND DISCUSSION

In order to get insight into the mechanisms involved in silver accumulation, the *in situ* speciation of silver accumulated by *C. actinabiotes* was investigated. Algae exposed to $10^{-6}$ M, $10^{-5}$ M, $10^{-4}$ M, and $10^{-3}$ M Ag$^+$ accumulated respectively 51, 520, 2500, and 6500 µg$_{Ag}$/g$_{fw}$, and were analyzed using XAS at the silver K edge. Two trends clearly appear for silver oxidation state and chemical environment depending on whether microalgae are exposed to low or high silver concentration.

The XANES spectra of algae samples were compared to the reference spectra of silver foil (Ag(0)) and Ag$_2$S in Figure 1. The XANES of the sample exposed to $10^{-6}$ M and $10^{-5}$ M Ag$^+$ are similar and display two weak oscillations separated by a plateau at ca. 25.52 and 25.54 keV. On the contrary, the samples exposed to $10^{-4}$ M and $10^{-3}$ M Ag$^+$ display two distinct oscillations at ca. 25.52 and 25.55 keV. From a qualitative comparison and despite the poor spectral resolution at this edge (about 6 eV from the core hole at this edge), it appears that the first set of spectra resembles that of the Ag$_2$S reference, whereas the second set is similar to the Ag foil reference. One may therefore assume that the environment of silver accumulated by the algae at low concentrations is comparable to that in Ag$_2$S whereas it is mostly reduced to Ag(0) at higher concentrations.

The EXAFS spectra are presented in Figure 2A and their corresponding Fourier transforms in Figure 2B, together with the fitted curves. As observed with the above XANES data, EXAFS spectra of the samples exposed to $10^{-4}$ M and $10^{-3}$ M Ag$^+$ are very similar. They exhibit clear oscillations up to 14 Å$^{-1}$. The high frequency of those oscillations and their shape suggests the presence of heavy backscattering atoms like Ag. In contrast, the spectra of algae exposed to $10^{-5}$ M and $10^{-6}$ M Ag$^+$ are different from the previous set. Despite the EXAFS spectrum of the latter being very noisy (and considered up to only 8 Å$^{-1}$) due to low silver
concentration in the sample, both spectra show oscillations which are in phase and have comparable amplitudes.

At low silver concentration ($10^{-5}$ M), the Fourier transform of the EXAFS spectra exhibit two main contributions, at pseudo-distances of $R + \Phi = 1.9$ Å and 2.75 Å. The spectra were successfully fitted (R factor = 3%) using two independent shells of Ag-S and Ag-Ag. Ag-O and Ag-N contributions were also tested and were ruled out because the corresponding distances were aberrant. These contributions correspond respectively to 2.0(1) S atoms located at an average distance of 2.41(1) Å and 0.9(1) Ag atoms located at 2.93(1) Å (Table 1). These fitted parameters are in good agreement with typical values reported in the literature for *in vitro* systems implying Ag and sulfur containing ligands. Silver EXAFS spectrum of Cd$_2$Ag$_{17}$-metallothioneins complexes has been for instance reported to be successfully fitted using 2 S atoms at 2.40 Å and an Ag contribution (without any indication of the coordination number) at 2.9 Å.\textsuperscript{32} Ag-S average distances of 2.40(2) Å were also observed in Ag(+I)-penicillamine solutions.\textsuperscript{33} Our results also indicate the presence of both two S in the first Ag coordination shell and one Ag neighbor. Sulfur neighbors are expected in ligands such as phytochelatins or metallothioneins. Nonetheless, the presence of an Ag neighbor in the structure is surprising and would indicate the occurrence of at least Ag dimers in the structure. Another assumption to explain this presence would be the concomitant formation of Ag nanoparticles (see below), although in very minor proportion, that would intermix with the presence of the sulfur compound. In any case these results suggest that Ag$^+$ ions accumulated by *C. actinabiots* when exposed to low concentrations react with cellular molecules and complex to sulfur containing groups. This is consistent with the preferential chemical interaction between silver(I) ions and thiol-containing species.\textsuperscript{34,35} Free Ag$^+$ ions are complexed by thiol-containing species such as cysteine, glutathione, metallothioneins or phytochelatins known to be involved in metal detoxification mechanisms. *In vivo*, silver has
been shown to bind both high-molecular-weight substances and metallothioneins in the hepatocytosol of animals\textsuperscript{36} and to induce phytochelatin synthesis in microalgae\textsuperscript{37}. In the context of bioremediation, the change in silver speciation enables the immobilization of the metallic ion inside the \textit{C. actinabiotis} cells in a non-toxic form and preserves the cellular functioning as will be shown by the toxicity experiments presented below. This is the main phenomenon occurring at silver concentrations representative of that contained in industrial effluents which are of the order of $10^{-6}$-$10^{-5}$ M.

At higher silver concentrations ($10^{-4}$ M and $10^{-3}$ M), we observed from the EXAFS, XRD, and TEM results the formation of silver metal nanoclusters inside the algae cells themselves. The EXAFS spectra were strongly dominated by Ag-Ag backscattering, which accounted for the contributions at pseudo-distances of $R + \Phi = 2.7$, 3.9, 4.9, and 5.5 Å on the Fourier transform (Figure 2B). All these peaks were successfully fitted using the fcc silver model described in the experimental section. The first Ag-Ag distances are equal to 2.87(2) and 2.87(1) Å and average coordination numbers equal to 3.6(1) and 3.9(1) for samples exposed to $10^{-4}$ M and $10^{-3}$ M, respectively. These distances are remarkably consistent with an Ag(0) fcc phase as described in the experimental section (list of all the paths in Table S1 of the Supporting Information). It is also in agreement with distances reported for silver nanoclusters embedded in glass (2.88(1) Å at 15 K)\textsuperscript{38} or synthesized by living alfalfa sprouts (2.88 Å).\textsuperscript{39} The low value of the Ag-Ag coordination numbers (respectively 3.6(1) and 3.9(1)) compared to the fcc structure (coordination number equals to 12) suggests a deviation from a pure fcc phase and the presence of silver nanoclusters within the algae, as observed previously using TEM.\textsuperscript{16} It is actually known that small metal clusters have smaller average coordination numbers than bulk structures because the atoms located at the surface of the cluster have less neighboring atoms, which causes the decrease of the average coordination number. Montejano-Carrizales and co-workers reported a formula that links the average
coordination number of a cluster to its diameter, assuming the cluster is rather small (10-1000 atoms) and spherical.\textsuperscript{40} When applying this formula to the present results, an fcc silver cluster with an average coordination number of about 4 corresponds to a particle diameter of the order of few nms. However one might keep in mind that an average coordination number of 4 may also be obtained when numerous small clusters of a few silver atoms or even isolated atoms are present together with some large clusters of few tens of nm. Such low Ag-Ag coordination number has already been observed in the case of Ag nanoparticles embedded in a silica glass and attributed to the presence of Ag-O contributions.\textsuperscript{41}

Indeed, beside the strong Ag-Ag interactions, the Fourier transform of the EXAFS spectra of samples exposed to high silver concentrations exhibit a zone of disagreement at a pseudo-distance of ca. $R + \Phi = 2$ Å. Although this zone is strongly affected by the lobes of the Fourier transform of the Ag-Ag scattering, we attempted to fit this zone with an additional low $R$ contribution. At first the presence of O neighbors has been tested and gave a satisfactory agreement in this zone. This corresponds to a distance of ca. 2.20 Å. Several oxygenated biological ligands have actually been shown to bind silver.\textsuperscript{34} Dubiel and co-workers fitted similar spectra with Ag-O contributions at 2.13-2.14 Å when studying silver speciation in silicate glasses.\textsuperscript{41} However, the longer distance obtained in our study makes the hypothesis of O neighbors as a first shell unlikely. Instead this contribution may be fitted using an additional Ag-S shell that significantly improved the fit (R factor from 0.039 to 0.019). It corresponds to 0.7(1) S at a distance of 2.46(1) Å. This bond length is in agreement with values reported for silver bound to rabbit liver metallothioneins MT1 (2.45(2) and 2.44(3) Å for Ag\textsubscript{12}-MT1 and Ag\textsubscript{17}-MT1, respectively).\textsuperscript{42} It is also comparable with the Ag-S distance determined for silver concentration equal to $10^{-5}$ M. However the very low coordination number suggests considering this additional contribution with care because the
interference between a minority of Ag-S and a majority of Ag-Ag backscatters at such low R
distance value is strongly influenced by the amplitude of the latter.

Given the above data, it can be assumed that the reduced silver nanoparticles described
above coexist with unreduced isolated silver species (silver ions bound to thiols for instance)
as observed for the lowest concentrations. In addition, part of the surface atoms of the
nanoparticles may be linked to sulfur atoms (or much less likely to oxygen atoms) capping
and stabilizing the nanoparticles. This suggests that the algae accumulate Ag(+I) sulfur
species (like at low silver concentrations) but reduce Ag$^+$ into Ag(0) nanoparticles when the
silver exposure increases. It is likely that these different mechanisms coexist.

The crystallinity of the silver nanoparticles formed inside algae was further
investigated using X-Ray diffraction after freeze-drying. The diffractogram of control algae
exposed to no silver showed that the spectra baseline is distorted because of the amorphous
phases present in the freeze-dried algae and of the kapton® film. The diffractogram of algae
exposed to 10$^{-2}$ M Ag$^+$ displays noticeable peaks at 2θ = 38.1°, 44.3°, 64.4°, and 77.5° (Figure
3), corresponding to the fcc silver reference diffraction pattern (planes (111), (200), (220), and
(311), respectively), which clearly indicates the existence of fcc silver nanoparticles inside the
algae. Silver crystals probably form independently from any light processes as they appeared
in microalgae exposed to Ag$^+$ in the light as well as in the dark (Figure 3). The three most
intense peaks (111), (220), and (311) obtained on the diffractogram were fitted assuming a
pseudo-Voigt shape function. A Scherrer type analysis lead to a mean crystallite size of 10 ± 2
nm. But the peak shape indicates the presence of a broad distribution. These results are
consistent with previous TEM observation of similar samples which highlighted the presence
of clusters containing silver as revealed by energy dispersive spectroscopy analysis$^{16}$, and
whose size ranged from some nm to about 30 nm.$^{16}$ This is compatible also with the EXAFS
data at $10^{-4}$ M and $10^{-3}$ M. It demonstrates the fcc structure of in situ synthesized silver nanoparticles by *Coccomyxa actinabiots*. The synthesis of silver nanoparticles by dead or living biomass, including plant extracts\textsuperscript{43,44}, plants\textsuperscript{39}, bacteria\textsuperscript{45} and recently the green microalga *Chlamydomonas reinhardtii*\textsuperscript{46}, has been reported. However, the crystallinity of these particles had not been assessed in microalgae. When crystallinity was assessed, plant extract were shown to yield two kinds of silver crystal structures, namely hexagonal and fcc\textsuperscript{47}. The fcc crystals obtained here in *Coccomyxa actinabiots* are thermodynamically the most stable configuration compared to hexagonal structures which are metastable.\textsuperscript{48,49}

Nanoparticles localization and size were examined using TEM in microalgae exposed to $10^{-4}$ M and $10^{-2}$ M Ag\textsuperscript{+}. At $10^{-4}$ M, nanoparticles appear mainly in the chloroplast and in mitochondria (Figure 4A,B). The distribution of particle size is monodisperse, ranging from 4 to 28 nm, with most particles in the 6 to 12 nm range (Supplementary Figure S2A). The presence of even smaller particles cannot be excluded but is difficult to infer from the images. The biggest clusters may correspond to agglomerates of smaller nanoparticles. The plastid localization agrees with Beattie and co-workers observations on living plants showing that silver nanoparticles are most abundant in the chloroplasts of *Brassica juncea* exposed to $2 \times 10^{-2}$ M Ag\textsuperscript{+}.\textsuperscript{50} However, in *Chlamydomonas reinhardtii* exposed to $10^{-3}$ M Ag\textsuperscript{+}, nanoparticles are localized in the peripheral cytoplasm and at flagella root.\textsuperscript{46} The discrepancy between both green microalgae may be due to different stages of nanoparticles formation related to different response thresholds to silver.

When silver intracellular concentration increases, silver nanoparticles appear in almost all cellular compartments, namely in the chloroplast, in mitochondria, in the cytosol along the plasma membrane, on membrane structures such as Golgi apparatus and in vacuoles (Figure 4C,D). This localization is consistent with previous observations using nano X-ray
fluorescence. The distribution of particle size is comparable to that observed at lower silver concentrations, with most particles in the 6 to 12 nm range (Supplementary S2A) and is identical whatever the subcellular cell compartment, whether being the chloroplast, mitochondria or vacuoles (Supplementary Figure S2B). This distribution is consistent with the mean particle size of 10 nm evaluated by X-ray diffraction in algae exposed to $10^{-2}$ M Ag. The somewhat higher size of some clusters may result from the aggregation of crystallites or coalescence of smaller nanoparticles as observed in Figure 4D and reported by Gardea-Torresdey and co-workers.

The formation of silver nanoparticles actually implies the reduction of Ag$^+$ and the nucleation of the metallic seeds, their growth with the aggregation of these small nuclei and their stabilization by capping molecules like oxygen, nitrogen, or sulfur containing molecules. In microalgae, their formation probably relies on different simultaneous mechanisms, several molecules being able to be implied in Ag$^+$ reduction and nanoparticles stabilization. Various enzymes such as ATP synthase, superoxide dismutase, carbonic anhydrase and ferredoxin-NADP$^+$ reductase have been shown to be associated with in vivo synthesis of silver nanoparticles in *Chlamydomonas reinhardtii*. Plant extracts containing aldehyde, amino and carboxyl functions as well as the chemical species oxygen superoxide $O_2^-$ have also been demonstrated to synthesize silver or gold nanoparticles or assist their synthesis. Moreover, owing to the high reducing potential of Ag$^+$ of 0.8V, many reducing metabolites such as NADH, NADPH, glutathione or ferredoxin, whose respective redox potential are about -0.32V, -0.32V, -0.22V, and -0.43V, are thermodynamically able to reduce Ag$^+$. The initial localization of silver nanoparticles of substantial size in the chloroplast and mitochondria may result from several favorable dispositions. First, these compartments contain high amounts of adequate reducing metabolites such as ferredoxin and
NADPH in plastids and NADH, FADH₂, FMNH₂ in mitochondria. Secondly, the chloroplast contains enzymes shown to be associated with nanoparticle formation as mentioned above, such as ferredoxin-NADP⁺ reductase, carbonic anhydrase, superoxide dismutase, and ATP synthase, the latter two being also present in mitochondria. It is noteworthy that nanoparticles seem to form in intermembrane mitochondrial space rather than in mitochondrial matrix (Figure 4). A high local free Ag⁺ concentration, together with favorable reduction and nucleation conditions, probably favors the formation of particles of substantial size in intermembrane space, whereas their formation in matrix is probably hampered by the low free Ag⁺ concentration owing to the very high metabolite and protein density, in the order of 400 mg mL⁻¹, or to limited Ag⁺ passing through the membrane. Nanoparticle smaller size in microalgae compared to that obtained in different ex vivo conditions⁴⁷,⁵⁶ may be related to growth limitation owing to local Ag⁺ depletion as well as to capping by various soluble or membrane molecules.

The toxic effects of such silver concentrations to C. actinabiotis were examined. Silver toxicity was assessed both during and after exposure by monitoring algal growth and photosynthetic capacity. Growth actually reflects the overall physiological state of the cell including various fundamental physiological and biochemical processes, and the photosynthetic activity is the basis for cell development in these autotrophically-grown algae. Photosynthetic capacity was assessed through the measurement of the chlorophyll-a fluorescence yield Fᵥ/Fₘ.¹⁴ This parameter is very sensitive to metal toxicity.²⁴ As for silver speciation and subcellular localization, two different behaviours were brought out, depending on whether C. actinabiotis was exposed to low (≤ 10⁻⁵ M) or high (≥ 10⁻⁴ M) silver concentration (Supplementary Figure S1). During exposure to silver, the photochemical yield of microalgae contacted with low micromolar Ag⁺ concentrations did not change. An about
two fold reduction was noticed at 10^{-5} \text{ M} but the microalgae recovered their photosynthetic capacities within about four days when transferred into a silver-free growth medium (Supplementary Figure S1A). Contrastingly, the photochemical yield of microalgae contacted with higher Ag^{+} concentrations quickly dropped to zero, within 2 h, indicating that the photosynthetic apparatus was strongly damaged (Supplementary Figure S1A). The decrease in the F_{v}/F_{m} yield may result from Ag^{+} substitution to Cu^{+} in photosynthetic metalloproteins, both elements possessing close chemical reactivity. It was actually shown that the replacement of Cu^{+} by Ag^{+} in plastocyanine and cytochrome complex resulted in the inactivation of the photosynthetic electron transport.^{58} The ability of \textit{C. actinabiotis} to recover from silver exposure was further tested by transferring the microalgae into a silver-free culture medium. Low micromolar silver concentrations did not affect growth as compared to control (Supplementary Figure S1B). At the turning point of 10^{-5} \text{ M}, cells were able to actively detoxify silver and returned to a growth identical to that of control algae after a recovery period of about four days. Conversely, the photosynthetic apparatus in microalgae exposed to higher silver concentrations was irreversibly damaged. Their photochemical yield and their growth rate remained at zero (Supplementary Figures S1A and S1B). The photosynthesis inhibition could be sufficient to prevent cell growth; however, other processes may also be affected. The half maximum effective concentration (EC_{50}) of Ag^{+} toxicity in these conditions was therefore of the order of 10 \mu\text{M}, which is above toxicity values reported for other green microalga, though experimental conditions were different. EC_{50} of about 10-20 \text{ nM} and 25 \text{ nM} have actually been reported for growth inhibition of \textit{Chlamydomonas reinhardtii} and \textit{Pseudokirchneriella subcapitata}, respectively,^{59,60} and about 200 \text{ nM} for the inhibition of the photosynthetic yield in \textit{Chlamydomonas reinhardtii}.^{61} The somewhat higher resistance of \textit{C. actinabiotis} to silver toxicity makes it of interest for remediation processes based on a living biomass.
In conclusion, this work highlights the progressive change in processes implemented by *Coccomyxa actinabiotos* to take up and cope with silver when exposed to increasing Ag\(^+\) concentration. The simultaneous monitoring of silver *in situ* speciation and localization in relation to toxicity brought out two mechanisms, one prevailing at low micromolar Ag\(^+\) concentration, the other at high concentration, above 10\(^{-4}\) M Ag\(^+\). They are reported here together in living algae and plants for the first time, to the best of our knowledge. At low micromolar concentration, microalgae take up and sequester all silver initially present in solution, thus totally purifying the water. Silver is internalized in the cell and trapped in the cytosol\(^{16}\) mainly as unreduced Ag(+I) bound by sulfur containing molecules. It is likely that this chelation is the first mechanism that stops Ag\(^+\) diffusion inside the cell. This is probably part of an efficient detoxification process as the microalgal growth and photosynthetic activity actually remained unaffected. In the context of bioremediation, the knowledge of this mechanism provides the necessary information to optimize the sequestration capacities. This could be performed by genetic engineering to enhance the synthesis of these key metabolites and by providing the microalgae with adequate sulfate amounts. When silver external concentration increases, the stronger Ag\(^+\) intracellular influx overwhelms the sulfur complexation mechanisms. They prove insufficient to stop Ag\(^+\) in the cytosol and overcome its toxicity. Silver spreads throughout the cell. Most silver is reduced to Ag(0) and aggregates to form crystalline silver nanoparticles of about 10 nm, with a fcc structure. At 10\(^{-4}\) M Ag\(^+\), nanoparticles appear particularly in the chloroplast and mitochondria, organelles containing high reducing power and enzymes involved in oxidoreductive mechanisms. Nanoparticles formation may be one way to reduce silver toxicity once the complexation mechanisms by sulfur ligands are overwhelmed, as silver nanoparticles are less toxic than free Ag\(^+\).\(^{62}\) But even if it were the case, this process reveals insufficient or not quick enough to protect the
photosynthetic apparatus (the photochemical parameters collapsed when silver reaches the chloroplast) and prevent lethal effect beyond $10^{-4}$ M Ag+. When silver concentration still increases, nanoparticles continue to form, despite cell death, as observed with biomass extracts$^{44,52}$ and the amount of silver accumulated by microalgae continues to increase. Besides, interactions of silver with S still occur at high Ag+ concentration. Molecular mechanisms of Ag+ complexation and nanoparticles formation need further investigation. Nonetheless, even at $10^{-2}$ M Ag+, nanoparticles remain trapped inside microalgae, mostly embedded in intracellular membranes structures, which constitutes an asset for the bioremediation of contaminated effluents and environmental water. It is noteworthy that whatever the cellular mechanisms implemented (molecular chelation by sulfur groups or cluster formation), silver remains confined inside the cells.

The usual metal concentration limit in effluents issuing from the mining industry is around 1 mg.L$^{-1}$. In such conditions, the full silver load can be trapped into the algae. Our results also imply that effluent containing higher silver concentrations could be processed before being discharged into the environment, with similar remediation efficiency. Even higher concentrations will lead to the formation of silver nanoclusters and eventually to the algae cell death, but algae still constitute efficient bioremediators as nanoparticles will remain within the cells.

Further work will involve the application to polluted water, from e.g. aqueous effluents in the case of industrial applications, or fresh water stream in environmental application, using a bioreactor developed at laboratory pilot scale for other applications of this microalga$^{63}$. After depollution of the effluent by an algal suspension, the microalgae could be separated by micro-filtration or centrifugation. The fact that high amounts of silver are sequestered inside algae is of relevance to bioremediation as well as to recycling technologies. Recovery of silver from the collected algae could be considered as a new supply strategy.
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ASSOCIATED CONTENT

Supporting Information Available

Table S1. Best fit parameters from the refinement of the algae samples exposed to silver EXAFS spectra with the list of all the Ag-Ag distances included in the fitting procedure of algae samples exposed to silver at $10^{-3}$ and $10^{-4}$ M.

Figure S1. Physiological impact of silver on C. actinabiotis exposed to various Ag$^+$ concentrations and recovery thereafter including the changes in photosynthetic yield and algal growth.

Figure S2. Size distribution of nanoparticles in microalgae.

This information is available free of charge via the Internet at http://pubs.acs.org.
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### Table 1. Best fit parameters from the refinement of the algae samples exposed to silver EXAFS spectra.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ag-S</th>
<th>Ag-Ag</th>
<th>$S_0^2$, $\Delta e_0$, R factor, $\chi^2_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algae exposed to $10^{-3}$ M Ag$^+$ (15 K)</td>
<td>0.7(1) S at 2.46(1) Å $\sigma^2 = 0.0057$ Å$^2$</td>
<td>3.9(1) Ag at 2.87(1) Å $\sigma^2 = 0.0035$ Å$^2$</td>
<td>$S_0^2 = 0.9$, $\Delta e_0 = 2.53(30)$, R factor = 0.030, $\chi^2_R = 0.10$</td>
</tr>
<tr>
<td>Algae exposed to $10^{-4}$ M Ag$^+$ (15 K)</td>
<td>0.7(1) S at 2.46(1) Å $\sigma^2 = 0.0050$ Å$^2$</td>
<td>3.6(1) Ag at 2.87(2) Å $\sigma^2 = 0.0032$ Å$^2$</td>
<td>$S_0^2 = 0.9$, $\Delta e_0 = 1.73(24)$, R factor = 0.019, $\chi^2_R = 0.19$</td>
</tr>
<tr>
<td>Algae exposed to $10^{-5}$ M Ag$^+$ (15 K)</td>
<td>2.0(1) S at 2.41(1) Å $\sigma^2 = 0.0048$ Å$^2$</td>
<td>0.9(1) Ag at 2.93(1) Å $\sigma^2 = 0.0059$ Å$^2$</td>
<td>$S_0^2 = 0.7(1)$, $\Delta e_0 = 1.94(32)$, R factor = 0.030, $\chi^2_R = 0.19$</td>
</tr>
</tbody>
</table>

$\sigma^2$ is the Debye-Waller factor, $S_0^2$ the passive electron reduction factor, $\Delta e_0$ (in eV) the energy shift of the adjustment, R factor is the relative error of the fit versus data, and $\chi^2_R$ the reduced $\chi^2$ factor of the statistical chi-square test. Uncertainty is given on the last digit in brackets.
Figures

Figure 1. Ag K-edge normalized XANES spectra of algae exposed to $10^{-6}$ M, $10^{-5}$ M, $10^{-4}$ M and $10^{-3}$ M Ag$^+$ (solid lines) and of Ag reference compounds (Ag$_2$S for Ag(I) and Ag foil for Ag(0), bolted lines). The spectra have been vertically shifted for clarity.
Figure 2. (A) Ag K-edge $k^2$-weighted EXAFS spectra and (B) Fourier transforms moduli of algae exposed to $10^{-6}$ M, $10^{-5}$ M, $10^{-4}$ M and $10^{-3}$ M Ag$^+$. Solid line: experimental data; dotted line: best fit. The spectra have been vertically shifted for clarity.
Figure 3. X-ray diffraction data recorded for (A) solid line: microalgae exposed to $10^{-2}$ M Ag$^+$ in the light, (B) dotted line: microalgae exposed to $10^{-2}$ M Ag$^+$ in the dark, and (C) dashed line: reference sample of microalgae which were not exposed to silver. Vertical unit is arbitrary and spectra were vertically shifted for clarity. The blue lines indicate the peak positions expected for the silver Fm-3m crystallographic phase (face-centered cubic silver crystal) taken from file 00-004-0783 of the ICDD PDF-4 data base. In inset, fit of the (111) peak of the algal sample exposed to $10^{-2}$ M Ag$^+$ in the light assuming a pseudo-Voigt shape function.
Figure 4. Nanoparticles localization (black spots) in microalgae exposed to (A, B) $10^{-4}$ M and (C, D) $10^{-2}$ M Ag$^+$ using TEM. Chl, chloroplast; M, mitochondria; Cyt, cytosol; W, cell wall, S, starch granule; L, lipid vacuole; G, Golgi apparatus; PM, plasma membrane. Arrows point at a few silver clusters.
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