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Synchrotron XAS, XRD, and TEM**

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

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1 **ABSTRACT**

2

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

23

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

26 INTRODUCTION

27

28 Contamination of the environment by heavy metals and radionuclides is a world
29 concern which evolves with changing human uses. Among these contaminants, silver
30 constitutes one of the most toxic metals in aquatic environments, at the same level as
31 cadmium and chromium, surpassed only by mercury.¹ Silver toxicity depends on its
32 speciation, the free cationic form Ag^+ being highly toxic.^{1,2} Silver has long been used in the
33 photographic and imaging industry, in the electrical and electronics industry, in jewelry, in the
34 manufacture of silverware and coinage^{3,4} as well as for the treatment of diseases and
35 infections.⁵ Recently, applications of its antimicrobial properties strongly developed, silver
36 being now embedded in consumer products such as plasters, textiles, food containers,
37 toothpaste or air filters in the form of nanoparticles and colloids.⁶ Traditionally, silver is
38 discharged into the environment from e.g. mining and industrial applications⁴. But it is also
39 discharged nowadays from the normal use of consumer products which release silver
40 nanoparticles, colloids and Ag^+ .^{7,8} Additionally, silver also constitutes one of the main gamma
41 emitting radioactive contaminants present in liquid effluents issuing from nuclear pressurized
42 water reactors operating in normal conditions, representing up to 48% of the gamma emitting
43 radionuclides released.⁹

44 Remediation of effluents and of environmental water contaminated by heavy metals
45 and radionuclides is currently mostly performed using conventional physico-chemical
46 methods such as precipitation, oxidation/reduction or adsorption on ion-exchange resins.
47 These methods suffer from several drawbacks including cost, intolerance to organic species,
48 little efficiency for the removal of very low contaminant concentration and generation of large
49 secondary waste volumes.¹⁰ Biological remediation technologies based on organisms such as
50 bacteria, fungi and plants have offered competitive alternatives in various fields. They

51 generally present a high efficiency, a wider field of application and lower cost and impact on
52 the environment than physico-chemical technologies.^{11,12} Microalgae are good candidates for
53 heavy metal and radionuclide bioremediation strategies owing to their ability to fix a wide
54 range of contaminants and to resist to their chemical toxicity thanks to their large surface-to-
55 volume ratio, the structure of their cell wall carrying functional groups able to bind and
56 immobilize contaminants and various mechanisms enabling metal incorporation and
57 subcellular sequestration, excretion, or detoxification by speciation changes.¹³ Algae-based
58 biotechnologies for pollution remediation employ common green algae such as *Chlorella*,
59 *Scenedesmus*, *Cladophora*, cyanobacteria or consortia of both.¹⁴ Best metal accumulation
60 performance is obtained with microalgal species isolated from long-term metal contaminated
61 sites.¹³ Recently, a unicellular green microalga, *Coccomyxa actinabiotis*, was isolated from an
62 extreme environment contaminated with radioactive silver.¹⁵ High fixation capacities were
63 demonstrated for non-radioactive silver, namely 20 mg Ag g⁻¹ fresh weight (FW), *i.e.* about
64 200 mg Ag g⁻¹ dry weight (DW), among the higher values reported for various organisms.
65 When exposed to high Ag⁺ concentrations, up to 10⁻² M, most silver accumulated inside the
66 cell rather than on the mucilage surrounding the cell and the cell wall and formed silver
67 aggregates.¹⁶ *C. actinabiotis* showing also an extreme radiotolerance¹⁵, it is therefore an ideal
68 candidate in view to remove stable and radioactive silver both for remediation of
69 contaminated environmental water and for its recovery from industrial effluents.

70 The present study aims at exploring some of the molecular and cellular mechanisms of
71 silver accumulation and toxicity in *C. actinabiotis* in order to establish the potential of this
72 promising microalga for bioremediation. The fundamental understanding of the mechanisms
73 of silver uptake and toxicity constitutes the essential prerequisite for any biotechnological
74 applications. As a result, key parameters can be controlled and optimized. Silver speciation
75 was investigated directly *in situ* in microalgae suspended in Ag⁺ solutions ranging from μM to

76 mM concentrations. As mentioned above, metals in excess relative to the cellular metabolic
77 needs must be stored and/or their speciation modified to make them less toxic or facilitate
78 their transport. Reduction processes, chelation by specific peptides or proteins such as ferritin,
79 metallothioneins and phytochelatins or by small molecules may be involved.^{17,18} A variety of
80 analytical or spectroscopic methods have been used to study the speciation of metals in
81 biological and environmental media, such as UV-visible, infrared, Raman, X-ray Absorption
82 Spectroscopy (XAS), laser spectroscopy, mass spectrometry, nuclear magnetic resonance or
83 separation techniques followed by elemental analysis.^{19,20} XAS, comprising X-ray Absorption
84 Near-Edge Structure (XANES) and Extended X-ray Absorption Fine Structure (EXAFS)
85 spectra depending on the energy range above threshold, probes the near chemical environment
86 of the central cation within 4-5 Å, with a sensitivity of up to tens of ppm. Technological
87 advances made XAS suitable to study the speciation of metals also *in situ* in living
88 organisms.²¹ In this work, XAS (XANES and EXAFS), complemented by X-Ray diffraction
89 (XRD), were used to investigate the silver speciation in *C. actinabiotis*, highlighting various
90 oxidation states, coordination, and structure depending on the silver concentration. The
91 progressive formation and localization of silver nanoparticles was investigated at different
92 silver concentrations using electron microscopy (TEM), thus supplementing previous
93 localization work.¹⁶ Toxic effects of such Ag⁺ concentrations to *C. actinabiotis* were then
94 evaluated by monitoring physiological and biochemical parameters such as microalgal growth
95 and photosynthetic capacity which are commonly used to assess metal toxicity to
96 microalgae²²⁻²⁴. The combination of physiological, spectroscopic and physico-chemical
97 investigations using techniques such as XAS, XRD, and TEM revealed the implementation of
98 different cellular processes in the microalgae depending on silver concentration.
99 Considerations about the remediation capabilities in view to use *C. actinabiotis* for the clean-
100 up of contaminated water are presented.

101

102

103 **MATERIALS AND METHODS**

104

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

111 Microalgae were harvested in the growing phase by centrifugation (2000 g, 5 min,
112 4°C), washed by three successive re-suspensions in deionized water followed by
113 centrifugations (same conditions) to remove external elements coming from the culture
114 medium, and finally re-suspended in ultrapure water (MilliQ, Millipore) at $2 \text{ g}_{\text{FW}}\cdot\text{L}^{-1}$ to
115 perform the experiments. Algae were exposed to silver nitrate (AgNO_3 Carl Roth GmbH,
116 Karlsruhe, Germany) in ultrapure water rather than in culture medium in order to control
117 silver speciation, which was the free form Ag^+ in all experiments. The microalga *C.*
118 *actinabiotis* is known to grow naturally in ultra-pure water¹⁵ where it was discovered, so that
119 this medium change hardly affects its metabolism during the silver exposure protocol.
120 Particularly, the photochemical parameter F_v/F_m of control algae varied by less than 10% over
121 the time laps of exposure in pure water (Supplementary Figure S1A).

122

123 **X-ray Absorption Spectroscopy**

124 **Sample preparation.** The speciation of silver accumulated by microalgae was studied *in situ*
125 using XAS. Microalgae were exposed to 10^{-6} M, 10^{-5} M and 10^{-4} M Ag^+ for 16 h, and to 10^{-3}
126 M Ag^+ for 5 h, in polyethylene terephthalate glycol-modified (PETG) flasks, and incubated
127 in the light, temperature, and aeration conditions listed above. After exposure, algae were

128 harvested by centrifugation and washed three times with water (2000 g, 5 min, 4°C). Silver
129 concentration was determined in aliquots of pellet and supernatant by ICP-MS as described
130 below. Pellets, in the form of a thick paste, were placed in polypropylene sample holders,
131 sealed with Kapton®, plunged into liquid nitrogen, and kept at -80°C until analysis.

132

133 **XAS measurements.** Silver K-edge XANES and EXAFS spectra were collected at the
134 Rossendorf Beamline at the European Synchrotron Radiation Facility (ESRF, Grenoble,
135 France).²⁵ The beamline was equipped with a Si(111) double crystal monochromator,
136 operated in channel-cut mode, and calibrated at the Ag K-edge energy of a Ag metal foil. Two
137 Pt-coated Si mirrors were used to collimate the X-ray beam and achieve third-order
138 harmonics rejection. Spectra were collected at 15 K using a ⁴He cryostat. Samples were
139 measured in fluorescence mode using a 13-element high purity Ge detector (Canberra) and in
140 transmission mode using ionization chambers.

141

142 **XAS analysis.** XANES spectra were calibrated in energy using the Ag foil at 25514 eV
143 (first point in the derivative) and normalized using the Athena code.²⁶ Silver form in
144 the samples was qualitatively assessed by phenomenological comparison with
145 reference spectra, namely Ag foil for Ag(0) metallic silver and Ag₂S for Ag-S bonding
146 with formal oxidation state Ag(I). EXAFS data treatment was performed using the
147 Athena code after background subtraction and normalization with Ifeffit routine, and
148 then fitted using the Artemis code.²⁶ The EXAFS curves were extracted and k²-
149 weighted (k = wavenumber in Å⁻¹) for Fourier transformation between ca. 2.5 and
150 12 Å⁻¹. The fitting procedure was performed in real R space between 1 and 6 Å. Phases
151 and amplitudes were calculated using Feff84 code²⁷. For all Ag-Ag contributions, face-
152 centered cubic (fcc) metallic silver was used as a model compound (crystallographic

153 data from Kittel²⁸). 7 scattering paths corresponding to a cluster of 5.8 Å were selected
154 for the adjustment procedure: at 2.89 Å (2 legs), 4.09 Å (2 legs), 5.00 Å (2 legs), 5.39
155 Å (3 legs), and 5.78 Å (2, 3 and 4 legs). According to the fcc structure, all the paths were
156 linked together in the fitting procedure, so that only one Ag-Ag distance and one coordination
157 number were refined. When Ag-S contribution was also needed in the fit, it was
158 calculated using the structure of Ag₂S (crystallographic data from Frueh²⁹). The spectra
159 of samples exposed to low silver concentrations were best fitted using a combination
160 of Ag-S and Ag-Ag single shell contributions; whereas the spectra of samples exposed
161 to the higher silver concentrations were fitted with the previously specified seven Ag-
162 Ag contributions together with an additional Ag-S single shell contribution.

163

164 **X-ray diffraction.** Silver speciation at high concentration was complemented by XRD
165 measurements. Algae were exposed to 10⁻² M Ag⁺, in PETG flasks, for 3 h, in the light or in
166 the dark, in the conditions described above. After exposure, algae were harvested by
167 centrifugation and washed twice with water (2000 g, 5 min, 4°C). Pellets were frozen in liquid
168 nitrogen and kept at -80°C. Right before analysis, samples were freeze-dried and ground into
169 a fine powder. A drop of water was added. The obtained paste was homogeneously spread on
170 zero-background silicon sample holders, allowed to dry, and covered with a Kapton® film. A
171 control sample (algae not exposed to silver) was prepared in the same conditions to measure
172 the algal baseline. Measurements were performed in Bragg-Brentano geometry, on a
173 PANalytical X'Pert diffractometer equipped with an X'Celerator linear detector, at $\lambda = 1.54$ Å
174 (Cu K_α emission lines). International Centre for Diffraction Data (ICDD) database
175 (<http://www.icdd.com>) was used to identify peaks; datasheet 00-004-0783 was used as
176 reference for fcc silver. To determine the crystallite size, the three most intense peaks were
177 fitted assuming a pseudo-Voigt shape function. A Scherrer type analysis³⁰ was performed

178 considering the resulting full widths at half maximum corrected from the instrumental
179 resolution.

180

181 **Transmission electron microscopy.** Silver localization in microalgae exposed to 10^{-4}
182 M and 10^{-2} M Ag^+ was analyzed using TEM as described in Leonardo *et al.* 2014.¹⁶ To
183 determine the nanoparticle size distribution, the size of all particles in a given subcellular
184 compartment or in a given section of a compartment was measured.

185

186 **Inductively-coupled plasma-mass spectrometry.** Silver uptake was assessed directly
187 from silver concentration in microalgae using an inductively-coupled plasma-mass
188 spectrometry (ICP-MS) apparatus described previously³¹ at $m/z = 107$ and 109 , using
189 calibration solutions in 1% HNO_3 (v/v). Prior to ICP-MS analysis, algae pellets were
190 mineralized by suspension in 5 mL of concentrated HNO_3/HCl (2/1 v/v) (Suprapur® HNO_3
191 and HCl , Merck, Darmstadt, Germany) for 3 h at 170°C before dilution to 1% HNO_3 (v/v).

192

193 **Silver toxicity assessment.** Silver toxicity was assessed by monitoring the maximum
194 quantum yield of photosystem II F_v/F_m^{24} and the cellular growth during silver exposure and
195 recovery. After exposure for 2 h to 10^{-7} M to 10^{-3} M Ag^+ in the conditions described above, in
196 the light, microalgae were harvested by centrifugation, washed twice with water (2000 g,
197 5 min, 4°C) and allowed to recover in the culture conditions described above.

198

199

200

201 **RESULTS AND DISCUSSION**

202

203 In order to get insight into the mechanisms involved in silver accumulation, the *in situ*
204 speciation of silver accumulated by *C. actinabiotis* was investigated. Algae exposed to
205 10^{-6} M, 10^{-5} M, 10^{-4} M, and 10^{-3} M Ag^+ accumulated respectively 51, 520, 2500, and 6500
206 $\mu\text{g}_{\text{Ag}}\cdot\text{g}_{\text{FW}}^{-1}$, and were analyzed using XAS at the silver K edge. Two trends clearly appear for
207 silver oxidation state and chemical environment depending on whether microalgae are
208 exposed to low or high silver concentration.

209 The XANES spectra of algae samples were compared to the reference spectra of silver
210 foil ($\text{Ag}(0)$) and Ag_2S in Figure 1. The XANES of the sample exposed to 10^{-6} M and 10^{-5} M
211 Ag^+ are similar and display two weak oscillations separated by a plateau at ca. 25.52 and
212 25.54 keV. On the contrary, the samples exposed to 10^{-4} M and 10^{-3} M Ag^+ display two
213 distinct oscillations at ca. 25.52 and 25.55 keV. From a qualitative comparison and despite the
214 poor spectral resolution at this edge (about 6 eV from the core hole at this edge), it appears
215 that the first set of spectra resembles that of the Ag_2S reference, whereas the second set is
216 similar to the Ag foil reference. One may therefore assume that the environment of silver
217 accumulated by the algae at low concentrations is comparable to that in Ag_2S whereas it is
218 mostly reduced to $\text{Ag}(0)$ at higher concentrations.

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

226 concentration in the sample, both spectra show oscillations which are in phase and have
227 comparable amplitudes.

228 At low silver concentration (10^{-5} M), the Fourier transform of the EXAFS spectra
229 exhibit two main contributions, at pseudo-distances of $R + \Phi = 1.9 \text{ \AA}$ and 2.75 \AA . The spectra
230 were successfully fitted (R factor = 3%) using two independent shells of Ag-S and Ag-Ag.
231 Ag-O and Ag-N contributions were also tested and were ruled out because the corresponding
232 distances were aberrant. These contributions correspond respectively to 2.0(1) S atoms
233 located at an average distance of 2.41(1) \AA and 0.9(1) Ag atoms located at 2.93(1) \AA (Table
234 1). These fitted parameters are in good agreement with typical values reported in the literature
235 for *in vitro* systems implying Ag and sulfur containing ligands. Silver EXAFS spectrum of
236 $\text{Cd}_2\text{Ag}_{17}$ -metallothioneins complexes has been for instance reported to be successfully fitted
237 using 2 S atoms at 2.40 \AA and an Ag contribution (without any indication of the coordination
238 number) at 2.9 \AA .³² Ag-S average distances of 2.40(2) \AA were also observed in Ag(+I)-
239 penicillamine solutions.³³ Our results also indicate the presence of both two S in the first Ag
240 coordination shell and one Ag neighbor. Sulfur neighbors are expected in ligands such as
241 phytochelatins or metallothioneins. Nonetheless, the presence of an Ag neighbor in the
242 structure is surprising and would indicate the occurrence of at least Ag dimers in the structure.
243 Another assumption to explain this presence would be the concomitant formation of Ag
244 nanoparticles (see below), although in very minor proportion, that would intermix with the
245 presence of the sulfur compound. In any case these results suggest that Ag^+ ions accumulated
246 by *C. actinabiotis* when exposed to low concentrations react with cellular molecules and
247 complex to sulfur containing groups. This is consistent with the preferential chemical
248 interaction between silver(I) ions and thiol-containing species.^{34,35} Free Ag^+ ions are
249 complexed by thiol-containing species such as cysteine, glutathione, metallothioneins or
250 phytochelatins known to be involved in metal detoxification mechanisms. *In vivo*, silver has

251 been shown to bind both high-molecular-weight substances and metallothioneins in the
252 hepatocytosol of animals³⁶ and to induce phytochelatin synthesis in microalgae³⁷. In the
253 context of bioremediation, the change in silver speciation enables the immobilization of the
254 metallic ion inside the *C. actinabiotis* cells in a non-toxic form and preserves the cellular
255 functioning as will be shown by the toxicity experiments presented below. This is the main
256 phenomenon occurring at silver concentrations representative of that contained in industrial
257 effluents which are of the order of 10^{-6} - 10^{-5} M.

258 At higher silver concentrations (10^{-4} M and 10^{-3} M), we observed from the EXAFS,
259 XRD, and TEM results the formation of silver metal nanoclusters inside the algae cells
260 themselves. The EXAFS spectra were strongly dominated by Ag-Ag backscattering, which
261 accounted for the contributions at pseudo-distances of $R + \Phi = 2.7, 3.9, 4.9,$ and 5.5 \AA on the
262 Fourier transform (Figure 2B). All these peaks were successfully fitted using the fcc silver
263 model described in the experimental section. The first Ag-Ag distances are equal to $2.87(2)$
264 and $2.87(1) \text{ \AA}$ and average coordination numbers equal to $3.6(1)$ and $3.9(1)$ for samples
265 exposed to 10^{-4} M and 10^{-3} M, respectively. These distances are remarkably consistent with an
266 Ag(0) fcc phase as described in the experimental section (list of all the paths in Table S1 of
267 the Supporting Information). It is also in agreement with distances reported for silver
268 nanoclusters embedded in glass ($2.88(1) \text{ \AA}$ at 15 K)³⁸ or synthesized by living alfalfa sprouts
269 (2.88 \AA).³⁹ The low value of the Ag-Ag coordination numbers (respectively $3.6(1)$ and $3.9(1)$)
270 compared to the fcc structure (coordination number equals to 12) suggests a deviation from a
271 pure fcc phase and the presence of silver nanoclusters within the algae, as observed
272 previously using TEM.¹⁶ It is actually known that small metal clusters have smaller average
273 coordination numbers than bulk structures because the atoms located at the surface of the
274 cluster have less neighboring atoms, which causes the decrease of the average coordination
275 number. Montejano-Carrizales and co-workers reported a formula that links the average

276 coordination number of a cluster to its diameter, assuming the cluster is rather small (10-1000
277 atoms) and spherical.⁴⁰ When applying this formula to the present results, an fcc silver cluster
278 with an average coordination number of about 4 corresponds to a particle diameter of the
279 order of few nms. However one might keep in mind that an average coordination number of 4
280 may also be obtained when numerous small clusters of a few silver atoms or even isolated
281 atoms are present together with some large clusters of few tens of nm. Such low Ag-Ag
282 coordination number has already been observed in the case of Ag nanoparticles embedded in
283 a silica glass and attributed to the presence of Ag-O contributions.⁴¹

284 Indeed, beside the strong Ag-Ag interactions, the Fourier transform of the EXAFS
285 spectra of samples exposed to high silver concentrations exhibit a zone of disagreement at a
286 pseudo-distance of ca. $R + \Phi = 2 \text{ \AA}$. Although this zone is strongly affected by the lobes of
287 the Fourier transform of the Ag-Ag scattering, we attempted to fit this zone with an additional
288 low R contribution. At first the presence of O neighbors has been tested and gave a
289 satisfactory agreement in this zone. This corresponds to a distance of ca. 2.20 \AA . Several
290 oxygenated biological ligands have actually been shown to bind silver.³⁴ Dubiel and co-
291 workers fitted similar spectra with Ag-O contributions at 2.13-2.14 \AA when studying silver
292 speciation in silicate glasses.⁴¹ However, the longer distance obtained in our study makes the
293 hypothesis of O neighbors as a first shell unlikely. Instead this contribution may be fitted
294 using an additional Ag-S shell that significantly improved the fit (R factor from 0.039 to
295 0.019). It corresponds to 0.7(1) S at a distance of 2.46(1) \AA . This bond length is in agreement
296 with values reported for silver bound to rabbit liver metallothioneins MT1 (2.45(2) and
297 2.44(3) \AA for Ag₁₂-MT1 and Ag₁₇-MT1, respectively).⁴² It is also comparable with the Ag-S
298 distance determined for silver concentration equal to 10⁻⁵ M. However the very low
299 coordination number suggests considering this additional contribution with care because the

300 interference between a minority of Ag-S and a majority of Ag-Ag backscatters at such low R
301 distance value is strongly influenced by the amplitude of the latter.

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

309

310 The crystallinity of the silver nanoparticles formed inside algae was further
311 investigated using X-Ray diffraction after freeze-drying. The diffractogram of control algae
312 exposed to no silver showed that the spectra baseline is distorted because of the amorphous
313 phases present in the freeze-dried algae and of the kapton® film. The diffractogram of algae
314 exposed to 10⁻² M Ag⁺ displays noticeable peaks at $2\theta = 38.1^\circ$, 44.3° , 64.4° , and 77.5° (Figure
315 3), corresponding to the fcc silver reference diffraction pattern (planes (111), (200), (220), and
316 (311), respectively), which clearly indicates the existence of fcc silver nanoparticles inside the
317 algae. Silver crystals probably form independently from any light processes as they appeared
318 in microalgae exposed to Ag⁺ in the light as well as in the dark (Figure 3). The three most
319 intense peaks (111), (220), and (311) obtained on the diffractogram were fitted assuming a
320 pseudo-Voigt shape function. A Scherrer type analysis lead to a mean crystallite size of 10 ± 2
321 nm. But the peak shape indicates the presence of a broad distribution. These results are
322 consistent with previous TEM observation of similar samples which highlighted the presence
323 of clusters containing silver as revealed by energy dispersive spectroscopy analysis¹⁶, and
324 whose size ranged from some nm to about 30 nm.¹⁶ This is compatible also with the EXAFS

325 data at 10^{-4} M and 10^{-3} M. It demonstrates the fcc structure of *in situ* synthesized silver
326 nanoparticles by *Coccomyxa actinabiotis*. The synthesis of silver nanoparticles by dead or
327 living biomass, including plant extracts^{43,44}, plants³⁹, bacteria⁴⁵ and recently the green
328 microalga *Chlamydomonas reinhardtii*⁴⁶, has been reported. However, the crystallinity of
329 these particles had not been assessed in microalgae. When crystallinity was assessed, plant
330 extract were shown to yield two kinds of silver crystal structures, namely hexagonal and fcc⁴⁷.
331 The fcc crystals obtained here in *Coccomyxa actinabiotis* are thermodynamically the most
332 stable configuration compared to hexagonal structures which are metastable.^{48,49}

333

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

346 When silver intracellular concentration increases, silver nanoparticles appear in almost
347 all cellular compartments, namely in the chloroplast, in mitochondria, in the cytosol along the
348 plasma membrane, on membrane structures such as Golgi apparatus and in vacuoles (Figure
349 4C,D). This localization is consistent with previous observations using nano X-ray

350 fluorescence.¹⁶ The distribution of particle size is comparable to that observed at lower silver
351 concentrations, with most particles in the 6 to 12 nm range (Supplementary S2A) and is
352 identical whatever the subcellular cell compartment, whether being the chloroplast,
353 mitochondria or vacuoles (Supplementary Figure S2B). This distribution is consistent with the
354 mean particle size of 10 nm evaluated by X-ray diffraction in algae exposed to 10^{-2} M Ag.
355 The somewhat higher size of some clusters may result from the aggregation of crystallites or
356 coalescence of smaller nanoparticles as observed in Figure 4D and reported by Gardea-
357 Torresdey and co-workers.³⁹

358

359 The formation of silver nanoparticles actually implies the reduction of Ag^+ and the
360 nucleation of the metallic seeds, their growth with the aggregation of these small nuclei and
361 their stabilization by capping molecules⁵¹ like oxygen, nitrogen, or sulfur containing
362 molecules. In microalgae, their formation probably relies on different simultaneous
363 mechanisms, several molecules being able to be implied in Ag^+ reduction and nanoparticles
364 stabilization. Various enzymes such as ATP synthase, superoxide dismutase, carbonic
365 anhydrase and ferredoxin-NADP⁺ reductase have been shown to be associated with *in vivo*
366 synthesis of silver nanoparticles in *Chamydomonas reinhardtii*.⁴⁶ Plant extracts containing
367 aldehyde, amino and carboxyl functions^{44,52} as well as the chemical species oxygen
368 superoxide O_2^- ⁵³ have also been demonstrated to synthesize silver or gold nanoparticles or
369 assist their synthesis. Moreover, owing to the high reducing potential of Ag^+ of 0.8V, many
370 reducing metabolites such as NADH, NADPH, glutathione or ferredoxin, whose respective
371 redox potential are about -0.32V, -0.32V, -0.22V, and -0.43V⁵⁴, are thermodynamically able
372 to reduce Ag^+ . The initial localization of silver nanoparticles of substantial size in the
373 chloroplast and mitochondria may result from several favorable dispositions. First, these
374 compartments contain high amounts of adequate reducing metabolites such as ferredoxin and

375 NADPH in plastids and NADH, FADH₂, FMNH₂ in mitochondria. Secondly, the chloroplast
376 contains enzymes shown to be associated with nanoparticle formation as mentioned above,
377 such as ferredoxin-NADP⁺ reductase, carbonic anhydrase, superoxide dismutase, and ATP
378 synthase, the latter two being also present in mitochondria. It is noteworthy that nanoparticles
379 seem to form in intermembrane mitochondrial space rather than in mitochondrial matrix
380 (Figure 4). A high local free Ag⁺ concentration, together with favorable reduction and
381 nucleation conditions, probably favors the formation of particles of substantial size in
382 intermembrane space, whereas their formation in matrix is probably hampered by the low free
383 Ag⁺ concentration owing to the very high metabolite and protein density, in the order of 400
384 mg·mL⁻¹,⁵⁵ or to limited Ag⁺ passing through the membrane. Nanoparticle smaller size in
385 microalgae compared to that obtained in different *ex vivo* conditions^{47,56} may be related to
386 growth limitation owing to local Ag⁺ depletion as well as to capping by various soluble or
387 membrane molecules.

388

389 The toxic effects of such silver concentrations to *C. actinabiotis* were examined. Silver
390 toxicity was assessed both during and after exposure by monitoring algal growth and
391 photosynthetic capacity. Growth actually reflects the overall physiological state of the cell
392 including various fundamental physiological and biochemical processes, and the
393 photosynthetic activity is the basis for cell development in these autotrophically-grown algae.
394 Photosynthetic capacity was assessed through the measurement of the chlorophyll-a
395 fluorescence yield F_v/F_m .²⁴ This parameter is very sensitive to metal toxicity.^{23,57} As for silver
396 speciation and subcellular localization, two different behaviours were brought out, depending
397 on whether *C. actinabiotis* was exposed to low ($\leq 10^{-5}$ M) or high ($\geq 10^{-4}$ M) silver
398 concentration (Supplementary Figure S1). During exposure to silver, the photochemical yield
399 of microalgae contacted with low micromolar Ag⁺ concentrations did not change. An about

400 two fold reduction was noticed at 10^{-5} M but the microalgae recovered their photosynthetic
401 capacities within about four days when transferred into a silver-free growth medium
402 (Supplementary Figure S1A). Contrastingly, the photochemical yield of microalgae contacted
403 with higher Ag^+ concentrations quickly dropped to zero, within 2 h, indicating that the
404 photosynthetic apparatus was strongly damaged (Supplementary Figure S1A). The decrease
405 in the F_v/F_m yield may result from Ag^+ substitution to Cu^+ in photosynthetic metalloproteins,
406 both elements possessing close chemical reactivity. It was actually shown that the
407 replacement of Cu^+ by Ag^+ in plastocyanine and cytochrome complex resulted in the
408 inactivation of the photosynthetic electron transport.⁵⁸ The ability of *C. actinabiotis* to recover
409 from silver exposure was further tested by transferring the microalgae into a silver-free
410 culture medium. Low micromolar silver concentrations did not affect growth as compared to
411 control (Supplementary Figure S1B). At the turning point of 10^{-5} M, cells were able to
412 actively detoxify silver and returned to a growth identical to that of control algae after a
413 recovery period of about four days. Conversely, the photosynthetic apparatus in microalgae
414 exposed to higher silver concentrations was irreversibly damaged. Their photochemical yield
415 and their growth rate remained at zero (Supplementary Figures S1A and S1B). The
416 photosynthesis inhibition could be sufficient to prevent cell growth; however, other processes
417 may also be affected. The half maximum effective concentration (EC_{50}) of Ag^+ toxicity in
418 these conditions was therefore of the order of 10 μM , which is above toxicity values reported
419 for other green microalga, though experimental conditions were different. EC_{50} of about 10-
420 20 nM and 25 nM have actually been reported for growth inhibition of *Chlamydomonas*
421 *reinhardtii* and *Pseudokirchneriella subcapitata*, respectively,^{59,60} and about 200 nM for the
422 inhibition of the photosynthetic yield in *Chlamydomonas reinhardtii*.⁶¹ The somewhat higher
423 resistance of *C. actinabiotis* to silver toxicity makes it of interest for remediation processes
424 based on a living biomass.

425

426 In conclusion, this work highlights the progressive change in processes implemented
427 by *Coccomyxa actinabiotis* to take up and cope with silver when exposed to increasing Ag^+
428 concentration. The simultaneous monitoring of silver *in situ* speciation and localization in
429 relation to toxicity brought out two mechanisms, one prevailing at low micromolar Ag^+
430 concentration, the other at high concentration, above 10^{-4} M Ag^+ . They are reported here
431 together in living algae and plants for the first time, to the best of our knowledge. At low
432 micromolar concentration, microalgae take up and sequester all silver initially present in
433 solution, thus totally purifying the water. Silver is internalized in the cell and trapped in the
434 cytosol¹⁶ mainly as unreduced $\text{Ag}(+I)$ bound by sulfur containing molecules. It is likely that
435 this chelation is the first mechanism that stops Ag^+ diffusion inside the cell. This is probably
436 part of an efficient detoxification process as the microalgal growth and photosynthetic activity
437 actually remained unaffected. In the context of bioremediation, the knowledge of this
438 mechanism provides the necessary information to optimize the sequestration capacities. This
439 could be performed by genetic engineering to enhance the synthesis of these key metabolites
440 and by providing the microalgae with adequate sulfate amounts. When silver external
441 concentration increases, the stronger Ag^+ intracellular influx overwhelms the sulfur
442 complexation mechanisms. They prove insufficient to stop Ag^+ in the cytosol and overcome
443 its toxicity. Silver spreads throughout the cell. Most silver is reduced to $\text{Ag}(0)$ and aggregates
444 to form crystalline silver nanoparticles of about 10 nm, with a fcc structure. At 10^{-4} M Ag^+ ,
445 nanoparticles appear particularly in the chloroplast and mitochondria, organelles containing
446 high reducing power and enzymes involved in oxidoreductive mechanisms. Nanoparticles
447 formation may be one way to reduce silver toxicity once the complexation mechanisms by
448 sulfur ligands are overwhelmed, as silver nanoparticles are less toxic than free Ag^+ .⁶² But
449 even if it were the case, this process reveals insufficient or not quick enough to protect the

450 photosynthetic apparatus (the photochemical parameters collapsed when silver reaches the
451 chloroplast) and prevent lethal effect beyond 10^{-4} M Ag^+ . When silver concentration still
452 increases, nanoparticles continue to form, despite cell death, as observed with biomass
453 extracts^{44,52} and the amount of silver accumulated by microalgae continues to increase.
454 Besides, interactions of silver with S still occur at high Ag^+ concentration. Molecular
455 mechanisms of Ag^+ complexation and nanoparticles formation need further investigation.
456 Nonetheless, even at 10^{-2} M Ag^+ , nanoparticles remain trapped inside microalgae, mostly
457 embedded in intracellular membranes structures, which constitutes an asset for the
458 bioremediation of contaminated effluents and environmental water. It is noteworthy that
459 whatever the cellular mechanisms implemented (molecular chelation by sulfur groups or
460 cluster formation), silver remains confined inside the cells.

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

468 Further work will involve the application to polluted water, from e.g. aqueous
469 effluents in the case of industrial applications, or fresh water stream in environmental
470 application, using a bioreactor developed at laboratory pilot scale for other applications of this
471 microalga⁶³. After depollution of the effluent by an algal suspension, the microalgae could be
472 separated by micro-filtration or centrifugation. The fact that high amounts of silver are
473 sequestered inside algae is of relevance to bioremediation as well as to recycling technologies.
474 Recovery of silver from the collected algae could be considered as a new supply strategy.

475

476

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478

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485

486

487 **ASSOCIATED CONTENT**

488

489 **Supporting Information Available**

490

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

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

497 Figure S2. Size distribution of nanoparticles in microalgae.

498 This information is available free of charge via the Internet at <http://pubs.acs.org>.

499

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677

678 **Tables**

679

680 **Table 1. Best fit parameters from the refinement of the algae samples exposed to silver**681 **EXAFS spectra.**

Sample	Ag-S	Ag-Ag	S_0^2 , Δe_0 , R factor, χ^2_R
Algae exposed to 10^{-3} M Ag^+ (15 K)	0.7(1) S at 2.46(1) Å $\sigma^2 = 0.0057 \text{ \AA}^2$	3.9(1) Ag at 2.87(1) Å $\sigma^2 = 0.0035 \text{ \AA}^2$	$S_0^2 = 0.9$ $\Delta e_0 = 2.53(30)$ R factor = 0.030 $\chi^2_R = 0.10$
Algae exposed to 10^{-4} M Ag^+ (15 K)	0.7(1) S at 2.46(1) Å $\sigma^2 = 0.0050 \text{ \AA}^2$	3.6(1) Ag at 2.87(2) Å $\sigma^2 = 0.0032 \text{ \AA}^2$	$S_0^2 = 0.9$ $\Delta e_0 = 1.73(24)$ R factor = 0.019 $\chi^2_R = 0.19$
Algae exposed to 10^{-5} M Ag^+ (15 K)	2.0(1) S at 2.41(1) Å $\sigma^2 = 0.0048 \text{ \AA}^2$	0.9(1) Ag at 2.93(1) Å $\sigma^2 = 0.0059 \text{ \AA}^2$	$S_0^2 = 0.7(1)$ $\Delta e_0 = 1.94(32)$ R factor = 0.030 $\chi^2_R = 0.19$

682 σ^2 is the Debye-Waller factor, S_0^2 the passive electron reduction factor, Δe_0 (in eV) the energy683 shift of the adjustment, R factor is the relative error of the fit versus data, and χ^2_R the reduced684 χ^2 factor of the statistical chi-square test. Uncertainty is given on the last digit in brackets.

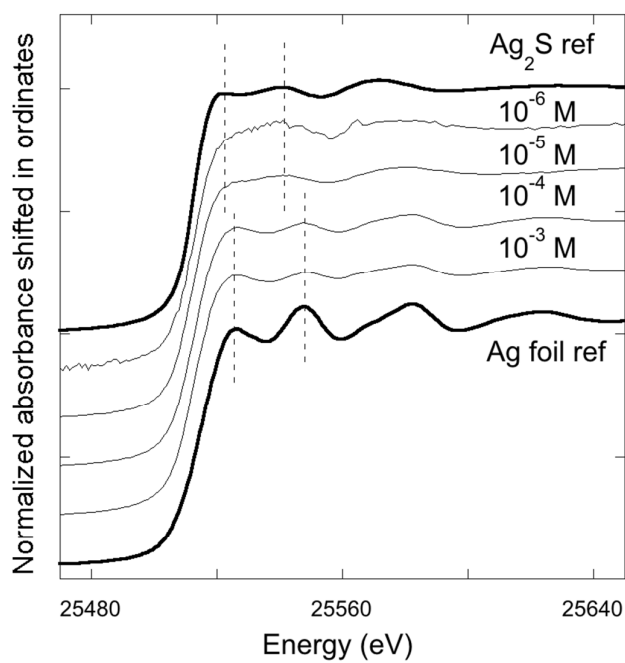
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686 **Figures**

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688 **Figure 1.** Ag K-edge normalized XANES spectra of algae exposed to 10^{-6} M, 10^{-5} M, 10^{-4} M
689 and 10^{-3} M Ag^+ (solid lines) and of Ag reference compounds (Ag_2S for Ag(I) and Ag foil for
690 Ag(0), bolted lines). The spectra have been vertically shifted for clarity.

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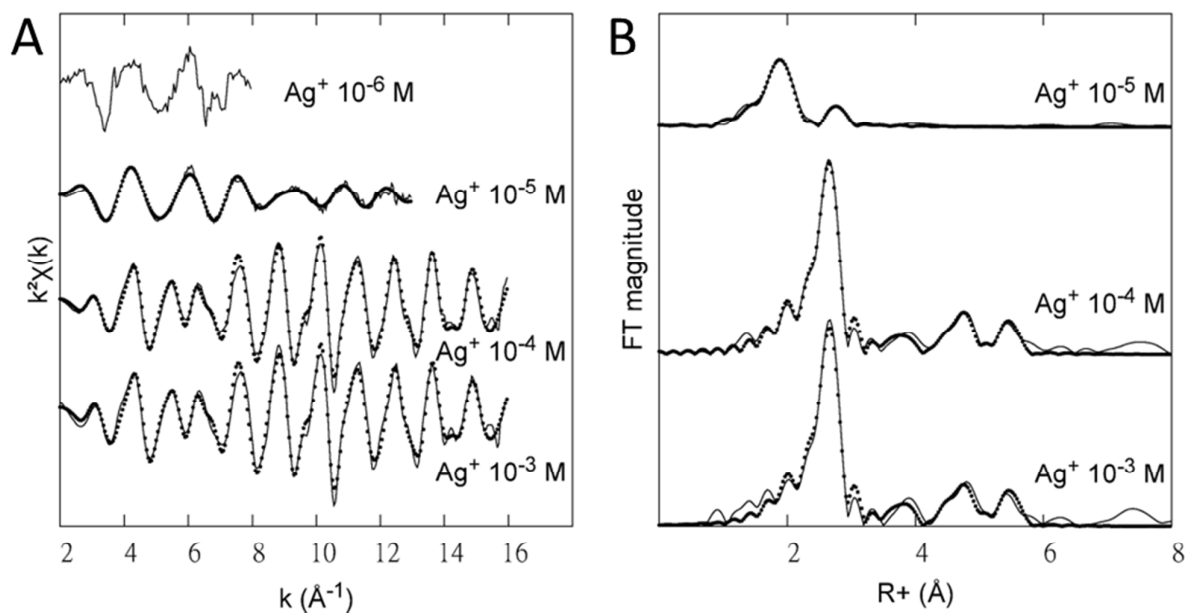


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694 **Figure 2.** (A) Ag K-edge k^2 -weighted EXAFS spectra and (B) Fourier transforms moduli of
695 algae exposed to 10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M Ag^+ . Solid line: experimental data; dotted
696 line: best fit. The spectra have been vertically shifted for clarity.

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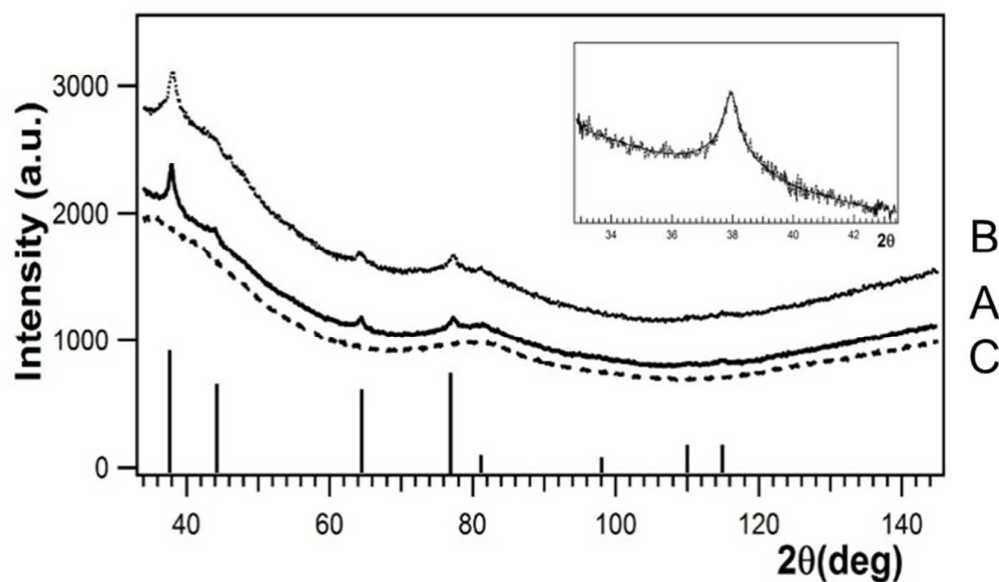


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700 **Figure 3.** X-ray diffraction data recorded for (A) solid line: microalgae exposed to 10^{-2} M
701 Ag^+ in the light, (B) dotted line: microalgae exposed to 10^{-2} M Ag^+ in the dark, and (C)
702 dashed line: reference sample of microalgae which were not exposed to silver. Vertical unit is
703 arbitrary and spectra were vertically shifted for clarity. The blue lines indicate the peak
704 positions expected for the silver Fm-3m crystallographic phase (face-centered cubic silver
705 crystal) taken from file 00-004-0783 of the ICDD PDF-4 data base. In inset, fit of the (111)
706 peak of the algal sample exposed to 10^{-2} M Ag^+ in the light assuming a pseudo-Voigt shape
707 function.

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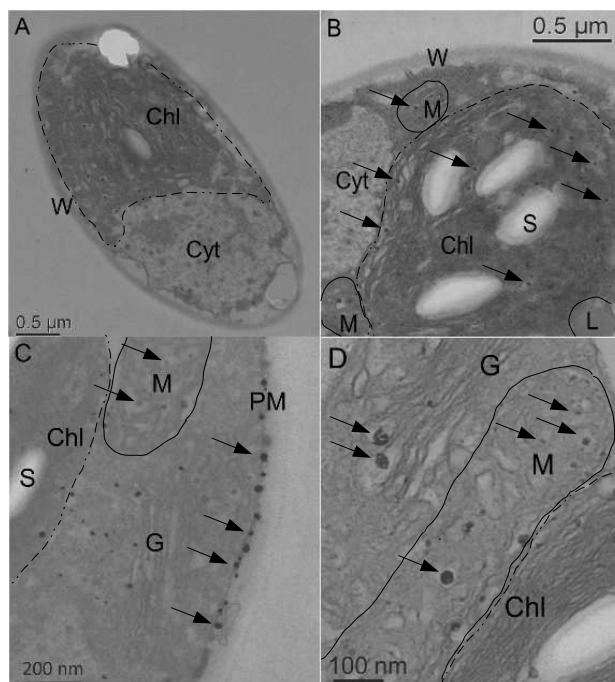


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711 **Figure 4.** Nanoparticles localization (black spots) in microalgae exposed to (A, B) 10^{-4} M and
712 (C, D) 10^{-2} M Ag^+ using TEM. Chl, chloroplast; M, mitochondria; Cyt, cytosol; W, cell wall,
713 S, starch granule; L, lipid vacuole; G, Golgi apparatus; PM, plasma membrane. Arrows point
714 at a few silver clusters.

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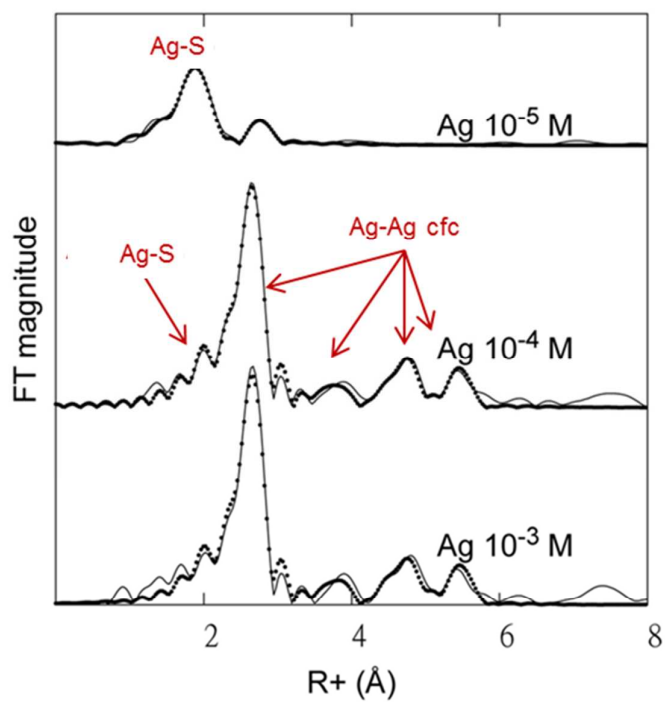
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