INFLUENCE OF CHLORIDE ON SILVER UPTAKE BY TWO GREEN ALGAE, *PSEUDOKIRCHNERIELLA SUBCAPITATA* AND *CHLORELLA PYRENOIDOSA*

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Abstract—Silver bioavailability in the presence of chloride was estimated from short-term (≤60 min) uptake experiments with two green algae, *Pseudokirchneriella subcapitata* and *Chlorella pyrenoidosa*. In the first experiment, silver uptake was monitored under two concentration regimes in which total dissolved silver ([Ag]tot) and [Cl] were manipulated to maintain [Ag+] at a constant value (10 nM). Comparable uptake rates were measured for both treatments despite the dramatic changes in [Cl] and [Ag]tot. In the second experiment, ambient [Ag]tot was held constant (10 or 115 nM), but [Cl] was varied (0.005–50 mM) to explore the whole range of silver chloro-complexes. Intracellular silver varied markedly along the [Cl] gradient and exhibited a clear, positive correlation with ambient [Ag+] for both algae. We conclude that the biotic ligand model reliably describes silver bioavailability in the presence of chloride for the two test algae and that its applicability depends on the relative magnitudes of silver fluxes through the unstirred diffusion layer and across the cell membrane, with the latter being affected by the presence or absence of a Cu(I) transporter. In the presence of chloride, no evidence was found for the internalization of silver via anion transport or passive diffusion of the neutral mono-chloro-complex, AgCP.

Keywords—Silver Speciation Diffusion Biotic ligand model Algae

INTRODUCTION

Freshwater algae play a key role in the biogeochemistry and ecotoxicology of silver. In surface waters, silver tends to associate with particulate matter, including algal cells (*K* from 104.5 to 106 [1]). Once assimilated by or adsorbed to algae, silver can be transferred either to the sediments by settling or to higher-level consumers through trophic pathways. In laboratory experiments, Ag uptake by consumption of various algal cells accounted for 4 to 49% of the total silver assimilation in zooplankton and bivalves [2–4]. In addition, zooplankton exhibited obvious signs of silver toxicity when silver was accumulated by dietary consumption of algal cells grown with environmentally relevant concentrations of silver [5]. In these latter experiments, toxic symptoms were not apparent when silver was taken up directly from water, even though body burdens of Ag in zooplankton were higher than in the case of dietary Ag uptake. Given this involvement of phytoplankton in silver biogeochemistry and ecotoxicology, it clearly is important to consider how silver interacts with algal cells.

In aquatic environments, three key factors should be known to fully comprehend a metal–organism interaction [6,7]: Metal speciation in the external environment, route of metal uptake by the organism, and biological effects of the metal on the organism. The speciation of silver is mostly influenced by the formation of strong complexes with simple inorganic ligands, such as thiosulfate or other sulfur(II)-containing ligands (formation constants, *K* ≈ 8.2), a behavior that is consistent with its class B, soft-metal characteristics [1]. In the absence of reduced sulfur species, silver reacts with simple, ubiquitous inorganic ligands, such as chloride, and forms less robust chloro-complexes (*K* ≈ 3.0). The biotic ligand model (BLM), a common model for metal–organism interactions, assumes that the free-ion concentration (or activity) of a metal ([M⁺]) is the primary factor determining dissolved metal uptake and toxicity [8] and, thus, predicts that the bioavailability of silver will be positively related to the concentration of the free ion, [Ag⁺], among various silver complexes in solution. The BLM, however, will apply only when a number of assumptions are satisfied. Two important assumptions are, first, that metal internalization, or transport of metal across the plasma membrane (*k*<sub>in</sub>) (Fig. 1), is slower than the advection or diffusion (*k*<sub>ex</sub>) of the metal toward the plasma membrane, and, second, that metal internalization occurs at cation-transport systems in the plasma membrane (Fig. 1) [7].

Earlier studies of the influence of inorganic ligands on silver–algal interactions raised some doubts about the ability of the BLM to predict silver uptake and toxicity [9–11]. For example, Fortin and Campbell [9] investigated the influence of chloride on silver uptake by a freshwater alga, *Chlamydomonas reinhardtii*, and demonstrated that silver uptake increased with the concentration of total silver and chloride, even though the free Ag⁺ concentration was fixed for all treatments. This result contradicted the BLM prediction that silver uptake rate would remain constant regardless of the [Cl] (or the total silver concentration) provided that the free Ag⁺ concentration remained unchanged. In this case, the failure of the BLM appeared to result from a rate of silver internalization that was greater than the diffusional flux from the bulk solution to the algal surface, a situation that is incompatible with the first assumption of the BLM. The BLM also failed to predict silver uptake by *C. reinhardtii* in the presence of another inorganic ligand, thiosulfate. Silver uptake was significantly faster in the presence of thiosulfate than in the presence of chloride, even though the free Ag⁺ concentration was the same in both treatments [10]. It was concluded that silver was accidentally transported across the plasma membrane via a sulfate/thiosulfate-
transport system in the form of silver–thiosulfate complexes and that this uptake via anion transport accounted for the accelerated silver uptake in the presence of thiosulfate.

In short, the initial studies of silver–algae interactions with *C. reinhardtii* have not conformed to the predictions of the BLM. Clearly, the need exists to expand the experimental database and to determine whether these initial results can be extrapolated to other algal species. In the present study, we have tested the ability of the BLM to predict short-term uptake of silver by unicellular algae in the presence of an inorganic ligand, chloride. For the uptake experiments, we have used two green algae, *Pseudokirchneriella subcapitata* and *Chlorella pyrenoidosa*, that internalize silver at slower rates than *Chlamydomonas reinhardtii* and, therefore, are more likely to satisfy the first assumption of the BLM.

**MATERIALS AND METHODS**

**Test organisms and culture preparation**

The experiments were performed with two green algae, *P. subcapitata* (formerly known as *Selenastrum capricornutum*; University of Toronto Culture Collection [UTCC] 37; UTCC, Toronto, ON, Canada) and *Chlorella pyrenoidosa* (UTCC 89) grown in a high-salt medium (Table 1, column 2) modified from that described by Macie et al. [12]. The medium was sterilized by autoclaving and then supplemented with filter-sterilized (pore size, 0.2 μm) to eliminate any fine particulates to which silver could adsorb, and then adjusted to pH 7.0 ± 0.1 (tolerated range, no pH buffers).

To minimize metal contamination, laboratory manipulations were carried out in containers made from Teflon®, polycarbonate, polypropylene, or polyethylene. Containers were soaked for at least 24 h in 10% HNO₃, thoroughly rinsed seven times with ultrapure water, and dried before use in a laminar flow hood under a positive pressure of filtered air. Reagents used in the present study were of analytical grade or better. Radioactivity of the silver isotope (¹¹⁰mAg; 136 mCi/mmol; Riso National Laboratory, Roskilde, Denmark) was measured on a liquid scintillation counter (1414 WinSpectral™; Wallac, Turku, Finland). Acidic stock solutions (0.2% HNO₃) of non-radioactive and radioactive silver were kept at less than pH 2 and at 4°C in the dark.

**Experimental procedures**

The experimental approach was designed to determine the silver uptake behavior of *P. subcapitata* and *C. pyrenoidosa* under two conditions: over a short period of time at a fixed [Ag⁺] and two different levels of the total [Ag] and [Cl], and over a wide range of [Cl] at a fixed total [Ag]. The two experiments are further explained in the following sections.

For the uptake experiments, algal cultures were initially inoculated with a small number of cells in the midexponential growth phase that were collected by gentle filtration (vacuum pressure, <10 cm Hg) onto a polycarbonate filter (pore size, 2.0 μm; Poretics, Minnetonka, MN, USA) trace metal mix to avoid metal precipitation. Batch cultures were grown axenically in 250-ml polycarbonate Erlenmeyer flasks (culture volume, 100 ml) under constant temperature at 20°C and illumination of approximately 100 μE/m²/s (cool white fluorescent lamps) with rotary agitation at 50 rpm. To identify the exponential growth phase, the batch cultures were monitored daily by removing a small aliquot (1 ml) and determining the culture densities using an electronic particle counter (model Multisizer™ 3; Coulter Electronics, Toronto, ON, Canada). Cultures were checked regularly for axenicity by plating on nutrient agar (Difco-Bacto agar, Liverpool, NSW, Australia).

**Reagents and glassware**

All exposure solutions were initially prepared from ultrapure water (18 MΩ/cm; Milli-QRO/Milli-Q2 system; Millipore, Bedford, MA, USA), which was filtered (polycarbonate filters; pore size, 0.2 μm) to eliminate any fine particulates to which silver could adsorb, and then adjusted to pH 7.0 ± 0.1 (tolerated range, no pH buffers).

Table 1. Composition of the algal culture medium (modified from Macie et al. [12]), the simplified culture-rinsing medium (without Ag), the simplified Ag-exposure medium, and the simplified culture-rinsing medium (with Ag).

<table>
<thead>
<tr>
<th>Components</th>
<th>Culture medium (mol/L)</th>
<th>Simplified culture-rinsing medium without Ag (mol/L)</th>
<th>Ag-exposure medium (mol/L)</th>
<th>Simplified culture-rinsing medium with Ag (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>5.98 × 10⁻⁶</td>
<td>5.00 × 10⁻⁶</td>
<td>Variable</td>
<td>1.00 × 10⁻⁷</td>
</tr>
<tr>
<td>Cl</td>
<td>4.22 × 10⁻³</td>
<td>4.00 × 10⁻³</td>
<td>4.00 × 10⁻³</td>
<td>4.00 × 10⁻¹</td>
</tr>
<tr>
<td>K</td>
<td>1.37 × 10⁻⁴</td>
<td>5.07 × 10⁻³</td>
<td>5.07 × 10⁻⁴</td>
<td>5.07 × 10⁻⁴</td>
</tr>
<tr>
<td>PO₄</td>
<td>1.02 × 10⁻⁴</td>
<td>5.00 × 10⁻⁶</td>
<td>5.00 × 10⁻⁴</td>
<td>5.00 × 10⁻⁴</td>
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*For ingredients common to all media and minor components for the culture media, refer to Fortin and Campbell [9].
metals. Algal cells were then resuspended in 10 ml of the same
simplified medium, and their size distribution, average surface
area, and culture density were determined using the electronic
particle counter. The resuspended cells were used for the silver-
exposure experiments (Table 1, column 4) under the conditions
outlined for each experiment. Silver exposure was carried out
for a short period of time (≤60 min) under ambient laboratory
conditions (22°C and a low-light regime of 7 μE/m²/s) at a
low culture density (10,000 cells/ml) to minimize the possible
effects of algal exudates on silver speciation. Measurements
of total silver concentrations at the end of a 60-min incubation
period showed that the algal cells had removed less than 8% of
the silver initially present in solution, indicating that silver
uptake had continued without ambient silver limitation during
the incubation period. Cells were then collected on two superimposed polycarbonate filters (pore size, 2.0 μm) in triplicates (the uptake experiments were run in triplicate, with three parallel incubations for any given set of experimental conditions), and the activity of the lower filter was subtracted from the measurement of the upper filter to correct for residual 110mAg radioactivity on filters. In the last step, to determine the true intracellular metal fraction, 110mAg adsorbed to the algal cell surface was removed by rinsing four times with 10 ml of simplified culture medium containing 100 nM non-radioactive Ag (Table 1, column 5). In our earlier studies [9,10], we had demonstrated the effectiveness of this treatment in removing adsorbed 110mAg. Silver speciation in the exposure solutions was computed with a chemical equilibrium program, MINEQL+ [13], using an updated thermodynamic database (www.inrs-ete.uquebec.ca/activites/groupes/biogeo/personal.htm). The thermodynamic data were derived from the default database supplied with the MINEQL+ software (Ver 4.5) but were updated by comparison with the National Institute of Standards and Technology Critical Stability Constants of Metal Complexes Standard Reference Database [14].

Test for silver uptake at fixed [Ag⁺] and variable total [Ag] and [Cl]

During a short period of time (≤60 min), silver uptake was
monitored in a medium with low concentrations of chloride
(5 μM) and total silver (10 nM) as well as in another medium
with high concentrations of chloride (4 mM) and silver (104
nM). In both exposure media, the free silver ion concentration
was kept at a constant level (10 nM). In the low-chloride
medium, almost all the silver exists in the form of the free
silver ion, whereas in the high-chloride medium, less than 10%
of the total silver remains as the free ion, with the majority
of the silver being present as the mono-chloro complex, AgCl⁰.
The ionic strength of both media was maintained at a constant
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Test for silver uptake at fixed total [Ag] and variable [Cl]

Silver uptake was monitored over a wide range of chloride
concentrations (0.005, 0.5, 5, and 50 mM) with the total silver
classification fixed at 10 or 115 nM. In this chloride concentra-
tion range, the dominant silver species undergo a major shift
(i.e., Ag⁺ dominant at 0.005 and 0.5 mM Cl, AgCl⁰ dominant
at 5 mM Cl, and AgCl₂⁻ at 50 mM Cl). Intracellular silver was
measured after a 15-min exposure to silver tracer at each chlor-
ide concentration. The relative contribution of individual sil-
Fig. 3. Short-term (15-min) silver uptake as a function of pCl at a fixed total silver concentration of 115 nM (symbols and connecting lines; right axis). The three curves without symbols represent the calculated distribution of silver–chloride complexes as a function of the chloride concentration (pCl or log[Cl]) (left axis; MINEQL+; Environmental Research, Hallowell, ME, USA). (A) Pseudokirchneriella subcapitata. (B) Chlorella pyrenoidosa. (C) Chlamydomonas reinhardtii (adapted from Fortin and Campbell [9]). Note the difference in scale of the y axis among species.

Fig. 4. Short-term (15-min) silver uptake as a function of pCl at a fixed total silver concentration of 10 nM (symbols and connecting lines; right axis). The three curves without symbols represent the calculated distribution of silver–chloride complexes as a function of the chloride concentration (pCl or log[Cl]) (left axis; MINEQL+; Environmental Research, Hallowell, ME, USA). (A) Pseudokirchneriella subcapitata. (B) Chlorella pyrenoidosa. (C) Chlamydomonas reinhardtii (adapted from Fortin and Campbell [9]). Note the difference in scale of the y axis among species.

medium. These rates were only approximately 6.3% (P. subcapitata) and 4.8% (C. pyrenoidosa) of the rate for Chlamydomonas reinhardtii (0.12 μmol Ag/m²/min) under identical conditions (Fig. 2C). In the low [Cl] medium, silver uptake rates were approximately 6.2 × 10⁻³ μmol Ag/m²/min for P. subcapitata and 4.2 × 10⁻³ μmol Ag/m²/min for Chlorella pyrenoidosa, which were approximately 20% (P. subcapitata) and 14% (C. pyrenoidosa) of the rate for Chlamydomonas reinhardtii (∼3.1 × 10⁻² μmol Ag/m²/min) (Fig. 2C). In our earlier study of C. reinhardtii [9], silver uptake was enhanced by a factor of 2.5 in the high [Cl] medium, even though [Ag⁺] was unchanged.

Silver uptake at fixed total [Ag] and variable [Cl] and [Ag⁺]

At an ambient total silver concentration of 115 nM, cell silver accumulation varied markedly for both P. subcapitata (Fig. 3A) and Chlorella pyrenoidosa (Fig. 3B) as the chloride concentration was increased. When the relative contribution of individual silver species to total silver uptake was estimated by partial correlation analysis, a clear, positive correlation was found between the intracellular silver content and the ambient [Ag⁺] for both algae (partial correlation coefficient, r = 0.969, p < 10⁻⁶ for P. subcapitata and r = 0.981, p < 10⁻⁶ for C. pyrenoidosa). Intracellular silver content, however, did not exhibit any positive relationships with the concentration of neutral AgCl⁰ (r = 0.245, p = 0.47 for P. subcapitata and r = 0.006, p = 0.99 for C. pyrenoidosa) or AgCl₂ (r = −0.237, p = 0.48 for P. subcapitata and r = 0.003, p = 0.99 for C. pyrenoidosa). Similar results were obtained in the low [Ag] medium; that is, silver uptake rates showed a positive correlation with ambient free Ag⁺ concentrations for both P. subcapitata (r = 0.245, p = 0.47 for P. subcapitata and r = 0.006, p = 0.99 for C. pyrenoidosa) or AgCl₂ (r = −0.237, p = 0.48 for P. subcapitata and r = 0.003, p = 0.99 for C. pyrenoidosa). In contrast, for Chlamydomonas reinhardtii (Figs. 3C and 4C), intracellular silver concentration decreased linearly with the increase of [Cl], and the uptake was not correlated with the concentration of either the free ion or the silver–chloride complexes [9].
Ability of the BLM to predict silver–algae interactions

In the present study, silver uptake was insensitive to a marked increase of the total silver concentration (10-fold increase) and chloride (800-fold increase) for both algae (Fig. 2A and B) provided that the ambient free Ag\(^+\) concentration remained constant. The slight difference in uptake rate between the two treatments appeared to result mainly from random variations among individual cultures used for the uptake assays. In general, silver uptake remained constant in conjunction with free silver ion concentration, as predicted by the BLM. This trend is confirmed by the uptake tests at fixed total [Ag] (Figs. 3A and B and 4A and B), which showed that silver uptake was correlated with the concentration of free silver ions rather than other silver–chloride complexes. These results corroborate the central idea of the BLM that the free silver ion concentration, or [Ag\(^+\)], determines the overall silver uptake rate.

These results differ markedly from those obtained earlier with C. reinhardtii, in which the BLM failed to predict silver uptake in the presence of chloride. This difference in behavior is consistent with the much lower silver uptake rates observed with P. subcapitata and Chlorella pyrenoidosa in the present study. For these two algal species, silver internalization appears to be the rate-limiting step, and the first assumption of the BLM is satisfied. For Chlamydomonas reinhardtii, however, silver internalization is more rapid, and diffusion of silver from the bulk solution to the algal surface becomes rate-limiting. We conclude that the BLM will apply to silver uptake by algae in the presence of chloride provided that the rate of silver internalization does not exceed the rate of diffusive supply of silver from the bulk solution.

Routes of silver internalization

The internalization of silver per se can be explained by the following three possible mechanisms (Fig. 1) [7,16]. First, because silver is not an essential element for growth, its uptake generally occurs via accidental cation transport or transport through a system used for the uptake of other essential cations (e.g., Na, K, or Cu(I)). Second, silver may cross cell membranes via passive diffusion directly through the lipid bilayer (i.e., as a neutral Ag\(^+\) complex). Finally, silver uptake may occur via accidental anion transport or transport via anion transporters as silver–anion complexes (e.g., as negatively charged silver–chloride complexes or silver–thiosulfate complexes).

Silver uptake via accidental cation transport. Silver(I) is accidentally transported through Cu(I)-transport systems in many prokaryotic and eukaryotic organisms, including bacteria, cyanobacteria, yeasts, and humans [17–21]. These Cu(I) transport systems are unable to differentiate Cu(I) from Ag(I), probably because both of them are group IB transition metals and, thus, share many physico-chemical properties, such as chemical reactivity, ionization energy, electronegativity, electron configuration, etc. In bacteria, cyanobacteria, and yeasts, P-type ATPases are involved in intracellular homeostasis of copper (and also silver); they remove excess intracellular Cu\(^+\) ion, which is potentially toxic, from the cytosol to the cell exterior (Escherichia coli, Enterococcus hirae, Candida albicans) [17,18,20] or translocate excess copper to the thylakoid interior (Synechococcus 7942) [19]. Recently, Solioz [22] suggested a comprehensive model for a plasma membrane copper uptake system, which is also responsible for silver uptake, in the bacterium E. hirae. Extracellular copper ions, mostly existing in the form of oxidized Cu(II) in solution, are first chemically reduced to Cu(I) by an extracellular cupric reductase. Copper(I) is then translocated into the cytoplasm by CopA, a P-type ATPase involved in Cu(I) uptake, and it can also be excreted from cytoplasm by CopB, another P-type ATPase. CopA was related to silver uptake based on an observation that a CopA knock-out strain was more silver-resistant than the wild type, probably because CopA was a path for silver into cells. Similarly, copper uptake in the yeast Saccharomyces cerevisiae occurs via a plasma membrane reductase [23] followed by a plasma membrane transporter (Ctr1) [24]. Cupric reductase activity was also reported in rabbit enterocytes [25] and tomato roots [26], and plasma membrane proteins analogous to yeast Ctr1 were found to be responsible for copper uptake in human embryonic kidney cells [21] and Arabidopsis thaliana [27].

In general, the concerted operation of cupric reductase and Cu(I) transporter appears to be a common characteristic of eukaryotic copper uptake pathways [28]. Interestingly, the cupric reductase appears to perform dual actions of iron reduction via the activity of ferric chelate reductase (FC-R), as well as cupric reduction, in a wide range of taxonomic groups, such as green algae [29], higher plants [26], and yeasts [23]. Therefore, activity of cupric reductase or FC-R may be used as an indication of the presence of a Cu(I) transporter in a given alga and for the accidental transport of Ag(I) via the transporter as well. For C. reinhardtii, both cupric reductase and transporter activities were found [28], and plasma membrane FC-R activity was reported for two Chlorella spp. [30,31], indicating the possibility of Ag(I) uptake via Cu(I) transporters in these algae. Although to our knowledge no evidence currently exists for reductive copper uptake in P. subcapitata, it is not irrational to assume its existence and involvement in silver uptake by this alga, considering the ubiquitous presence of the pathway.

Silver uptake via passive diffusion. In contrast to the present study, a number of reports in the literature have suggested that silver internalization may occur via passive diffusion of the neutral mono-chloro-complex. For example, Bury and Hogsstrand [32] recently reported that Ag\(^{3+}\) could be transported across the gill cell membrane of Atlantic salmon and rainbow trout yolk-sac fry. When silver accumulation was investigated as a function of the chloride concentration, it decreased along with [Ag\(^+\)], but the accumulation did not cease at high chloride concentrations, even though very little free Ag\(^+\) exists at these salinities. They also tested cell silver uptake as a function of ambient [Ag] at two fixed chloride concentrations, 0 mM (at which virtually all the silver is present as Ag\(^+\)) and 5 mM (at which Ag\(^{3+}\) comprises 62%, AgCl\(^-\) 31%, and Ag\(^+\) 7%). Silver uptake conformed to Michaelis-Menten kinetics at 0 mM [Cl] but increased linearly at 5 mM [Cl]. The linear increase of silver uptake at high [Cl] was interpreted as evidence for Ag\(^{3+}\) accumulation by passive diffusion.

These results are not necessarily inconsistent with ours. The seemingly different silver uptake behavior of fish may simply reflect the unique functional morphology of the salmonid gill, the entry point of silver into these organisms, rather than fundamentally different uptake mechanisms between algae and salmonid fish. For microalgae in the present study, virtually all cells in the culture are responsible for Ag\(^+\) uptake. However, for Atlantic salmon and rainbow trout, Ag\(^+\) uptake is limited to the specific ion-transport cells, whereas Ag\(^{3+}\) diffusion is presumably mediated by all types of gill cells. Because ion-
transport cells comprise only 5 to 10% of the total gill cells [33]. AgCl\(^{0}\) internalization via “ion-transport cells” may also comprise only 5 to 10% of the total observed AgCl\(^{0}\) internalization.

To normalize the comparison of salmonid fish with algae, the contribution of AgCl\(^{0}\) passive diffusion to total silver internalization was recalculated on a per-cell basis for the specific ion-transport cells in the salmonid gill. The salmonid data were recalculated in two steps. First, the AgCl\(^{0}\) diffusion rate was estimated from the difference between the total Ag uptake rate and the [Ag\(^{+}\)] uptake rate (i.e., AgCl\(^{0}\) diffusion rate = total Ag uptake rate − Ag\(^{+}\) uptake rate; the contribution of AgCl\(^{-}\) was considered to be negligible). When the Ag uptake rate at an ambient [Ag] of 115 nM (data point selected for comparison with our results in Fig. 3A and B) was interpolated from the Atlantic salmon data, the rate was approximately 80 pmol/g/h (in a medium with 0 mM [Cl]) or 20 pmol/g/h (in a medium with 5 mM [Cl]). Because Ag\(^{+}\) comprises 7% of the total silver at 5 mM [Cl], the Ag\(^{+}\) uptake rate would be approximately 7% of the maximum Ag\(^{+}\) uptake rate (i.e., the Ag\(^{+}\) uptake rate at 0 mM [Cl]). Therefore, in a medium with 5 mM [Cl], the Ag\(^{+}\) uptake rate can be estimated to be 5.6 pmol/g/h (80 pmol/g/h × 0.07) and the AgCl\(^{0}\) diffusion rate of all cells as 14.4 pmol/g/h (20 pmol/g/h [i.e., total Ag uptake rate] − 5.6 pmol/g/h [i.e., Ag\(^{+}\) uptake rate]). Second, the AgCl\(^{0}\) diffusion rate was calculated for the ion-transport cells using the AgCl\(^{0}\) diffusion rate of all gill cells and the mean proportion of ion-transport cells (7.5%, mean of 5% and 10%). In this manner, the AgCl\(^{0}\) diffusion rate for ion-transport cells was estimated as 1.08 pmol/g/h (14.4 pmol/g/h × 0.075), or only approximately 16% of the total Ag uptake rate (1.08 pmol/g/h [i.e., AgCl\(^{0}\) diffusion rate] + 5.6 pmol/g/h [i.e., Ag\(^{+}\) uptake rate] = 6.68 pmol/g/h); the passive diffusion rate was five orders of magnitude less than the internalization rate for C. pyrenoidosa (4.8 × 10\(^{-5}\) pmol/g/h) expressed on wet-weight basis (data not shown). This comparison is undoubtedly biased against the fish in that less cell surface area was available for passive diffusion per unit biomass because of the multicellular body structure, and the diffusion rate was calculated on the basis of the whole-body weight rather than on the gill cell weight only. Nevertheless, AgCl\(^{0}\) diffusion appears to be much slower than the internalization via accidental cation transporters in the alga, suggesting that even if AgCl\(^{0}\) diffusion occurred with the algal cells, it would be very difficult to detect.

Our findings are in striking contrast with the those of the earlier report by Reinfielder and Chang [11] that silver uptake rates of the marine diatom Thalassiosira weissflogii varied markedly as a function of the neutral AgCl\(^{0}\) concentration over the pCl range of 3.3 to 1.3 and exhibited its maximum level at a pCl of 2.3, at which neutral AgCl\(^{0}\) comprises the majority (~62%) of the total silver. In that study, AgCl\(^{0}\) alone appeared to correlate with silver uptake, and Ag\(^{+}\) did not show any positive relationship with the total silver uptake. The authors concluded that the alga accumulated AgCl\(^{0}\) as the principal silver–chloride complex, probably via passive diffusion. We speculate that the unusual behavior of T. weissflogii may stem from very slow silver uptake via cation transport, despite the existence in this alga of a membrane-bound Cu(I) transporter [34]; without appreciable Ag\(^{+}\) uptake via this route, overall silver internalization would be dominated by the AgCl\(^{0}\) diffusion pathway. This idea is supported by the observation that silver internalization is slower for the diatom than for the green algae at comparable chloride concentrations. At the peak of AgCl\(^{0}\) passive diffusion (i.e., pCl = 2.3; AgCl\(^{0}\) comprises 62% of the total silver), the concentration-specific silver internalization rate of T. weissflogii (2.4 × 10\(^{-8}\) mol/m\(^2\)/h/nM Ag) was slightly lower than that of C. pyrenoidosa (2.7 × 10\(^{-8}\) mol/m\(^2\)/h/nM Ag; calculated from Fig. 4B), even though the internalization rate of C. pyrenoidosa was at a lower level because of the low [Ag\(^{+}\)] (7% of the total silver). With the decrease of [AgCl\(^{0}\)] at a pCl of 3.3, the concentration-specific silver internalization rate of the diatom (6.3 × 10\(^{-9}\) mol/m\(^2\)/ h/nM Ag) declined to only approximately 17% of that of C. pyrenoidosa (3.8 × 10\(^{-8}\) mol/m\(^2\)/h/nM Ag).

Rate-limiting step in silver internalization

Besides the observational evidence presented above, estimations of the diffusional flux from the bulk solution to the algal surface corroborate the idea that the internalization of silver (k\(_{int}\)) (Fig. 1) is the rate-limiting step for these algae, as required by the BLM. The rate-limiting step in silver uptake can be determined by comparing the silver internalization rate with the flux of silver through the unstirred diffusion layer (k\(_{int}\)) (Fig. 1). In other words, as the observed internalization rate approaches the calculated maximum diffusion flux (the BLM assumes the internalization rate is much less than the maximum diffusion flux), silver uptake is more likely to be partly or fully limited by the diffusional flux; therefore, the BLM is less likely to be valid for describing metal uptake. The maximum diffusion rate (J\(_{Ag}^\prime\), expressed in pmol/cm\(^2\)/s) was calculated by the following equation [35]:

\[
J = \frac{4\pi D \left( \frac{r_c r_a}{r_a - r_c} \right) (C_h - C_i)}{A}
\]

where D is the diffusion coefficient of Ag\(^{+}\); r\(_c\) is the radius of the cell (4.4 × 10\(^{-4}\) cm for P. subcapitata and 5.3 × 10\(^{-4}\) cm for C. pyrenoidosa); r\(_a\) is the radius of the cell added to the thickness of the unstirred diffusion layer; C\(_h\) and C\(_i\) are the silver concentrations in the bulk solution (10 pmol/cm\(^2\)) and at the surface (assumed as 0 pmol/cm\(^2\)) for the maximum value of J\(_{Ag}^\prime\), respectively; and A is the algal surface area (0.8 × 10\(^{-6}\) cm\(^2\) for P. subcapitata and 1.20 × 10\(^{-6}\) cm\(^2\) for C. pyrenoidosa). We adopted the values for D (10 \(^{-8}\) cm/s) and the thickness of the diffusion layer (~8 × 10\(^{-4}\) cm; thus, r\(_a\) = 1.24 × 10\(^{-3}\) cm for P. subcapitata and 1.33 × 10\(^{-3}\) cm for C. pyrenoidosa) from the previous study [9] to facilitate a consistent comparison among different algal species.

The maximum diffusion flux was calculated as 1.4 pmol/ cm\(^2\)/s for P. subcapitata and 1.5 pmol/cm\(^2\)/s for C. pyrenoidosa, whereas the silver internalization rate was measured as 0.013 pmol/cm\(^2\)/s for P. subcapitata and 0.0095 pmol/cm\(^2\)/s for C. pyrenoidosa under comparable conditions. For both algae, the observed internalization rates were less than 1% of the calculated maximum diffusion flux (~0.9% for P. subcapitata and ~0.6% for C. pyrenoidosa); thus, it was highly unlikely that silver uptake was limited by diffusion flux of silver through the unstirred surface boundary layer. For C. reinhardtii [9], the internalization rate reached almost 10% of the maximum diffusion rate, indicating that the diffusion step could affect the internalization process.

CONCLUSION

The present study suggests that the BLM is a reliable tool for describing silver bioavailability in the presence of chloride
for two green algae, *P. subcapitata* and *C. pyrenoidosa*. The applicability of the BLM for the taxonomic group appeared to depend on the relative magnitudes of the flux of silver through the unstirred diffusion layer and the internalization flux across the cell membrane, with the latter being affected by the presence or absence of a Cu(I) transporter at the membrane level. However, in natural waters, where dissolved silver concentrations are much lower [1] than in our experimental system, silver uptake by algae may be limited simply by diffusional flux [36]. In such cases, the uptake rate will be directly proportional to the ambient dissolved silver concentration (see the maximum diffusion rate equation above). No evidence was found for the internalization of silver via anion transport or passive diffusion in the presence of chloride.

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