Labile synthetic cadmium complexes are not bioavailable to

***Pseudokirchneriella subcapitata* in resin buffered solutions**

L. Verheyen^{*}, R. Merckx and E. Smolders

Division of Soil and Water Management, K.U.Leuven, Kasteelpark Arenberg 20 - box 2459, 3001

Heverlee, Belgium. Liesbeth.Verheyen@ees.kuleuven.be; Erik.Smolders@ees.kuleuven.be

^{*}Corresponding author: Tel.: +32 16 32 16 30, fax: +32 16 32 19 97

E-mail address: Liesbeth.Verheyen@ees.kuleuven.be

Abstract

The Free Ion Activity Model (FIAM) predicts that cadmium (Cd) uptake by organisms is identical for solutions with the same free Cd^{2+} concentration and inorganic composition. Clear exceptions to the FIAM have been shown for Cd uptake by plant roots, periphyton and human cells where labile Cd complexes increase bioavailability and which has been attributed to their role in enhancing Cd diffusion towards the uptake cells. Here, we assessed the role of labile Cd complexes on Cd uptake by algae, for which diffusion limitations should be less pronounced due to their smaller size. Long-term (3 days) Cd uptake by the green algae *Pseudokirchneriella subcapitata* was measured in resin buffered solutions with or without synthetic ligands and at three Cd^{2+} ion activities (pCd 8.2-5.7). The free Cd^{2+} activity was maintained during the test using a metal-selective resin located in the algal bottles. Total dissolved Cd increased up to 35-fold by adding the synthetic ligands at constant Cd^{2+} activity. In contrast, Cd uptake by algae increased maximally 2.8 fold with increasing concentration of the synthetic ligands and the availability of the complexes were maximally 5.2% relative to Cd^{2+} for NTA and CDTA complexes. It is concluded that labile Cd complexes do not largely enhance Cd bioavailability to the unicellular algae and calculations suggest that Cd transport from solution to these small cells is not rate limiting.

Keywords: Metal uptake - Cadmium - Metal complexes - Green algae – FIAM-BLM

1 Introduction

The Free Ion Activity Model (FIAM) postulates that the uptake and toxicity of trace metals by organisms is related to the free ion activity of the metal in the solution. As a result, the addition of metal-complexing ligands to a solution of constant total dissolved metal decreases the bioavailability of the metal. The FIAM was probably first demonstrated for metal toxicity to algae (Anderson et al., 1978). An extension of the FIAM is the Biotic Ligand Model (BLM) that accounts for the ion competition effects at the biotic ligand. This model has been shown to be a powerful tool for predicting metal toxicity to biota in freshwaters varying in pH, hardness and DOM (Santore et al., 2002; De Schamphelaere and Janssen, 2006). The central assumption in the FIAM has been questioned in numerous studies showing that complexes may also contribute to metal uptake by biota (e.g. Bell et al., 1991; Smolders and McLaughlin, 1996). Direct uptake of intact metal complexes has been demonstrated, for example, for synthetic Pb complexes in plants (Schaider et al., 2006). Degryse et al. (2006 a, b) showed that uptake of Cd, Zn and Cu by plants in solutions, at identical free metal activity, was larger in presence than in absence of labile metal complexes. These effects were related to the contribution of labile complexes that overcome diffusive transport of the free metal ion from solution to the plant roots. A series of arguments were given to support this hypothesis. First, mass transfer limitations were illustrated by the larger Cd and Zn uptake in well stirred solutions compared to unstirred solution or agar, whereas no such effects were found in chelator buffered systems (Degryse et al., 2006c). Second, Cd uptake by plants correlated well with the diffusive Cd flux as measured with the DGT (diffusive gradient in thin films technique) across the solutions containing Cd complexes differing in lability (i.e. dissociate rate; Degryse et al., 2006a). Third, Cd, Zn and Cu uptake in all treatment was well predicted assuming diffusive transport only over a 0.1-0.4 mm unstirred layer

surrounding roots (Degryse et al., 2006a and b). Finally, interionic effects on metal uptake, e.g. pH or Ca^{2+} effects (cfr. BLM), were absent when mass transfer limits uptake, (i.e. transport and not membrane internalization controls uptake) but not in chelator buffered systems (Degryse and Smolders, 2012). The role of metal complexes on increasing metal bioavailability were also used to explain the deviations of the FIAM for explaining trace metals uptake in mussels (Jansen et al., 2002), Cd uptake in periphyton (Meylan et al., 2004; Bradac et al., 2009) or Cd and Zn uptake in Caco-2 cells (Verheyen et al., 2012b). Deviations of the FIAM are only rarely reported for metal uptake by algae and such may be related to smaller mass transport limitation. Indeed, small cells have larger surface area to weight ratio, and surface fluxes to sustain growth are, hence, smaller surface fluxes. Vigneault and Campbell (2005) confirmed the FIAM for short-term Cd uptake with algae in a series of solutions that contained either a synthetic ligand (NTA) or a natural organic ligand (Suwannee River fulvic or humic acid). The total Cd concentration was identical in the different solutions. No contribution of the Cd complexes was observed and the free Cd^{2+} ion appeared to be the preferred species for Cd uptake. In contrast, Fortin and Campbell (2000) found that uptake of Ag by a unicellular alga at fixed Ag^+ activity increased as the concentration of chloride (which forms AgCl° complexes with Ag^+), and total Ag concentration, increased. Calculations indicated that the Ag uptake was likely limited by diffusion, explaining the contribution of labile complexes to the uptake. Lamelas et al. (2007) observed a higher Pb internalization flux for freshwater algae in the presence of 10 mg L^{-1} humic acids than in the presence of $3 \times 10^{-4} \text{ M}$ citric acid at identical free Pb^{2+} concentrations. However, it is unknown if the speciation of the metals was controlled in these systems during exposure.

The evaluation of the FIAM critically relies on the metal speciation across treatments and during exposure. Predicted speciation is often not sufficient since stability constants lack sufficient accuracy. In addition, metal uptake can be a significant fraction of the available metal

when testing the FIAM for algae using the traditional algal bottle assay and algal exudates may alter metal speciation in solution. Recently, we developed and tested a resin buffered nutrient solution system for the algal bottle test using Cd (Verheyen et al., 2012a). A cation exchange resin located at the bottom of the beaker ensures that the Cd^{2+} is buffered during metal uptake and across treatments varying in concentration of ligands. At a fairly large cell density ($>10^6$ cells mL^{-1}), it was shown that Cd uptake obeyed the FIAM in the presence of the resin, but not in its absence, mainly, but not solely, because of the significant depletion of total dissolved Cd in the absence of the resin. That system was only tested for the effect of chloride (Cl^-) complexation of Cd (Verheyen et al. 2012).

The objective of this study is to assess if the FIAM is generally valid for Cd uptake by the algae *Pseudokirchneriella subcapitata* using the resin buffered exposure solutions to ensure a constant free metal ion concentration across treatments and during the exposure. Complexes stronger than the weak chloro-complexes were used to largely vary the ratio of complexed to free Cd^{2+} , also extending the range used by Vigneault and Campbell (2005). Long-term (72 hours) uptake experiments of Cd by the green algae *Pseudokirchneriella subcapitata* were performed in resin buffered systems with or without synthetic Cd complexes varying in lability. These treatments were applied at three different Cd^{2+} activities (pCd 8.2-5.7) since mass transport limitations are more likely at low than at high Cd^{2+} ion activity (Degryse and Smolders, 2012). The DGT labile metal was measured in these solutions to reflect the maximal diffusive flux in these systems. Chelex resin was used to buffer the Cd^{2+} in solution rather than the non-selective Amberlite resin used before by us because a strong buffer power was required in the solution with large concentrations of synthetic ligands; we have shown that Chelex also buffers Cd^{2+} , albeit at a slower rate than Amberlite (Verheyen et al. 2012a).

2 Materials and Methods

2.1 Test organism and culture conditions

All tests were conducted with the green algae *Pseudokirchneriella supcapitata*. The stock culture was obtained from the Culture Collection of Algae and Protozoa (CCAP 278/4, Windermere, UK). Upon arrival, the algae were transferred to 50 mL sterilized culture medium in 200 mL Erlenmeyer flasks. The culture medium is an adjusted WC-medium and contains 0.25 mM CaCl₂, 0.15 mM MgSO₄, 0.15 mM NaHCO₃, 65.4 μM K₂HPO₄, 0.5 mM NH₄NO₃, 5 mM HEPES (pH 7.4), 13 μM Na₄EDTA, 11.7 μM FeCl₃, 40 nM CuCl₂, 76.5 nM ZnSO₄, 42 nM CoCl₂, 0.91 μM MnCl₂, 23.4 nM Na₂MoO₄, and 16.2 μM H₃BO₃. The Erlenmeyer flasks were autoclaved for 20 minutes. After initiating the culture (cell density between 10⁴ and 10⁵ cells mL⁻¹), the flasks were closed with a cotton stopper. Every 7 days, cells were diluted in fresh medium to a cell density between 10⁴ and 10⁵ cells mL⁻¹. The algae were grown under a 12h/12h day/night cycle with illumination of 90 μE m⁻²s⁻¹ (cool white fluorescent lamps) and constant temperature of 21 ± 1°C. The cultures were continuously shaken with a horizontal shaker. Only algae in the exponential growth phase were used for the uptake experiments.

2.2 Preparation of the resin

The free Cd²⁺ activity in all test solutions was buffered by the resin Chelex[®]-100 (Bio-rad; 0.62 mmol_c (g wet weight)⁻¹) that was present in the algal uptake medium. The resin (4 g wet weight; Na-form) was first loaded by equilibrating the resin with a 50 mL Cd/Ca/Zn/Cu solution, all added as nitrate salts. Three different loadings were used to obtain three different Cd²⁺ activities. The equivalent proportions Cd:Ca:Zn:Cu were (i) 0.05:0.65:0.2:0.1, (ii) 0.2:0.5:0.2:0.1 and (iii) 0.65:0.05:0.2:0.1 by adding stoichiometric quantities of Cd, Ca, Zn and Cu to the resin,

assuming complete exchange. After 2 days equilibration (end-over-end equilibration), the resin was rinsed several times with ultrapure water (mQ) and a rinse solution containing 1 mM CaNO_3 , 0.25 mM MgSO_4 , 20 μM K_2HPO_4 , 0.1 mM K_2SO_4 and 5 mM HEPES (pH 7.4). All rinsed resins were suspended in 25 mL of that rinse solution (4 g resin per 25 mL), and 0.35 mL of this suspension with resin (or 0.056 g of resin) was added to 35 mL exposure solution (see below). The Cd^{2+} activity buffered by the resin in the nutrient solutions was determined by using GEOCHEM-PC (Parker et al., 1995) after equilibrating these resins with the synthetic ligand NTA (nitrilotriacetate) and analysis of total dissolved Cd. The loaded resins buffered the free Cd^{2+} at activities of (i) 6 nM (or pCd 8.2 ($=-\log$ free Cd^{2+} activity)), (ii) 30 nM (or pCd 7.5) and (iii) 2 μM (or pCd 5.7). The free Cu^{2+} and Zn^{2+} activity were equally determined by using GEOCHEM-PC. The pCu was 11.6 and pZn was 9.1.

2.3 *Experimental solutions*

Treatments were resin buffered systems, buffering Cd^{2+} at pCd 8.2, 7.5 or 5.7, with or without synthetic Cd complexes varying in lability. The concentrations of Cd ligands were selected to obtain similar total dissolved Cd concentrations at a constant free Cd^{2+} activity. (Table 1). The ligands were NTA; HEDTA: N-(2-hydroxyethyl)ethylenediamine-N,N',N'-triacetate; EDTA: ethylenediaminetetraacetate; EGTA: ethylene-bis-(oxyethylenenitrilo)-tetraacetate; CDTA: trans-1,2-cyclohexyldiamine-N,N,N',N'-tetraacetate. The concentrations were estimated by GEOCHEM-PC and ranged from 0.29 to 40 μM depending on ligand. A ligand-free control solution was included at each pCd value.

The exposure solution for the algae consists of a basal solution, loaded resin and a synthetic ligand (except for the control solutions). The basal solution contained 5mM HEPES, 1 mM $\text{Ca}(\text{NO}_3)_2$, 20 μM K_2HPO_4 , 0.25 mM MgSO_4 , 0.1 mM K_2SO_4 , 15 μM FeHBED, 0.3 μM MnCl_2 ,

3 μM H_3BO_3 , 60 nM CoCl_2 and 30 nM Na_2MoO_4 . The HEPES is a non-complexing buffer for Cd as confirmed with Donnan dialysis (details not shown). The metals Cu and Zn were not added as the equilibration with the Chelex provided for Cu and Zn in solution. Duplicate solutions were prepared (35 mL for 0.056 g resin) and equilibrated end-over end with the loaded resin for 5 days. After 5 days of equilibration, a subsample was taken for analysis with ICP-OES (Perkin Elmer, Optima 3300 DV). The exposure solutions were subsequently labelled with ^{109}Cd (533 kBq L^{-1} for the solutions with ligands and 5.33 MBq L^{-1} for the solutions without ligands) three days before their use.

2.4 *Metal uptake experiments*

The tests were performed in 100 mL beakers containing 35 mL of exposure solution. The beakers were covered with parafilm to reduce evaporation, but were not totally closed to allow gas exchange. There were two replicates for each treatment. Before inoculating the algae, the pH was measured and, if required, adjusted to pH 7.4 with NaOH or HCl. At the end of the exposure period the pH was measured again. The change in pH was negligible (< 0.1 unit). For inoculation, cells were harvested from the culture solution by centrifugation (4500 g, 15 min), and rinsed three times with a wash solution containing 5 mM CaCl_2 , 5 mM HEPES (pH 7.4) and 3 μM H_3BO_3 . The resuspended cells were inoculated into the uptake solutions for the test with synthetic ligands to obtain an initial cell density of circa 5×10^4 cells mL^{-1} . To determine the exact initial cell density, the cells were counted using a particle counter (HIAC Royco 9705). All tests were carried out under continuous illumination of 90 $\mu\text{E m}^2 \text{s}^{-1}$ (cool white fluorescent lamps) and a constant temperature of $21^\circ\text{C} \pm 1^\circ\text{C}$. The beakers were shaken three times a day. One mL of the test solution was sampled every day and analysed for ^{109}Cd with a gamma-counter (Packard Cobra autogamma) in the 15-40 keV energy window. The radioactivity concentration

was converted to molar units of Cd based on the measured initial specific activity (radioactivity relative to stable Cd concentration) in the test solution. After 3 days, 10 mL of the homogeneous test solutions with algae was sampled and collected. The sample was centrifuged for 15 minutes at 4500 g. One mL of the supernatant was analysed for ^{109}Cd , the supernatant was removed and the algae were rinsed with 5 mL of a solution containing 1 mM Na_4EDTA and 5 mM HEPES to remove adsorbed ^{109}Cd . After centrifugation and removal of the supernatant, the activity of ^{109}Cd in the algae, resuspended in 1 mL water was determined. Based on the specific activity of the solution, the molar quantity Cd taken up by the algae was calculated. Standard addition tests with aqueous spike solution showed 98% recovery of added ^{109}Cd in the resuspended cells. The algal density in each beaker was determined at the end of the experiment with the particle counter. The growth rate of the algae was calculated from the initial cell density (Q_{ini}) and the cell density (Q_{fin}) at the end of the assay (time t) as:

$$\mu = \ln(Q_{\text{fin}} / Q_{\text{ini}}) / t \quad (1)$$

The surface-based uptake flux, F_{uptake} , ($\text{mol cm}^{-2} \text{s}^{-1}$) was calculated from the amount of Cd taken up by the algae, M (mol mL^{-1}), taking growth into account

$$F_{\text{uptake}} = M \cdot \mu / (A_{\text{fin}} - A_0) \quad (2)$$

where A_0 and A_{fin} are the initial and final total algal surface area ($\text{cm}^2 \text{mL}^{-1}$), calculated from the measured cell density (cells mL^{-1}) and the surface area per cell ($= 5.03 \times 10^{-7} \text{cm}^2 \text{cell}^{-1}$ when spherical shape is assumed, OECD 201). Equation (2) was obtained by integration of: $dM = F_{\text{uptake}} \cdot A_t \cdot dt = F_{\text{uptake}} \cdot A_0 \cdot \exp(\mu \cdot t) \cdot dt$.

2.5 Diffusive Gradients in Thin Films (DGT)

The lability of the complexes was assessed using the DGT technique (Zhang and Davison, 2000). The DGT device consists of a chelating resin gel layer, overlaid by a hydrogel layer (diffusion layer) that is in contact with the solution and through which ions can freely diffuse. The concentration of DGT-labile species (c_{DGT}) is determined by measuring the mass of metal accumulated on the resin after a certain deployment time, and is proportional to the diffusional flux of metal through the hydrogel layer (F_{DGT}):

$$c_{\text{DGT}} = \frac{\Delta g \cdot F_{\text{DGT}}}{D} = \frac{\Delta g \cdot M}{D \cdot A \cdot t} \quad (3)$$

where M is the mass of metal accumulated on the resin, Δg (0.09 cm) is the thickness of the diffusion layer plus the thickness of the filter membrane, A is the surface area (3.14 cm²), D the diffusion coefficient (5×10⁻⁶ cm² s⁻¹), and t the deployment time. The contribution of the complexes to the DGT-flux is calculated as:

$$\xi_{\text{DGT}} = \frac{c_{\text{DGT}} - [\text{Cd}^{2+}]}{[\text{CdL}]} \quad (4)$$

If all complexes in solution fully dissociate within the DGT time scale, which depends on the thickness of the diffusion layer, c_{DGT} (measured on a stirred solution) equals the total concentration in solution, i.e. all species contribute equally to the diffusion flux ($\xi_{\text{DGT}}=1$). If the complexes do not dissociate at all within the DGT time scale, c_{DGT} equals the free ion concentration, i.e. only the free ion contributes to the diffusion flux ($\xi_{\text{DGT}}=0$).

The DGT technique was used on ¹⁰⁹Cd labeled solutions with synthetic ligands and a pCd of 8.2 but a total Cd concentration of 1 or 10 μM. The free Cd²⁺ activity of pCd 8.2 was obtained by equilibrating the solution with the chelex resin with the lowest fractional Cd²⁺ loading (0.05) for 2 days. The equilibrated solutions were filtered to obtain solutions free of chelex during the

experiment and labeled with ^{109}Cd (533 kBq L^{-1}) three days before the beginning of the test. The concentrations required for the synthetic ligands to obtain the total Cd concentrations of 1 and 10 μM were estimated with GEOCHEM-PC. The DGT devices, with a diffusion gel layer of 0.76 mm thickness, were placed (floating face down) in the radiolabeled uptake solutions that were placed on a horizontal shaker. After 22 hours, the DGT devices were removed, the resin gel was retrieved and ^{109}Cd activity of the gel was counted. The mass of (stable) Cd on the resin (M) was calculated from the ^{109}Cd activity and the specific activity of Cd in the solutions, which allowed calculating c_{DGT} (Eq. 3).

3 Results

3.1 *Solution speciation and lability of complexes assessed with DGT*

The concentrations of synthetic ligands were selected to obtain a total dissolved Cd concentration of ca. 0.1 (at $\text{pCd}=8.2$), 0.7 ($\text{pCd}=7.5$) or 10 μM ($\text{pCd}=5.7$) after equilibration with the metal-loaded Chelex resin (Table 1). Speciation calculations with GEOCHEM-PC were carried out to calculate the required ligand concentration, using the basal solution composition and the free ion activity (at which the resin buffered the solution) as input. The measured solution concentrations after equilibration with the resin were within a factor of two of the target concentrations indicating that the speciation calculations provide a reasonable prediction of the metal complexation.

The DGT technique was applied for solutions with synthetic ligands at $\text{pCd}=8.2$. Control deployment was carried out on a solution without Cd-complexing ligands, and the recovery (c_{DGT} predicted by Eqn. 3 relative to solution concentration) was 86%. In all solutions with complexes, c_{DGT} increased up to 589 fold compared to the ligand-free control (Figure 1). However, c_{DGT} was

smaller than the total concentration in solution, indicating that the complex did not fully dissociate within the diffusion layer. The contribution of the complex to the diffusion flux (ξ_{DGT}) at 1 μM total dissolved Cd followed the order NTA (0.57) > HEDTA (0.31) > EDTA (0.07) ~ EGTA (0.07) > CDTA (0.01), all these factors were significantly different ($p < 0.05$) except for CDTA. The results of the DGT experiment are in agreement with earlier DGT analyses and can be related to the difference in dissociation kinetics of these complexes assessed by other methods (Degryse et al. 2006a).

3.2 Uptake experiments in the presence of synthetic ligands

Algal growth in the presence of ligands was not significantly different ($p > 0.05$) from the ligand free controls at the corresponding pCd. The average growth rate of the two lowest Cd concentrations (pCd 8.2 and pCd 7.5) was 1.01 d^{-1} (standard error of 0.02). The growth rate at pCd 5.7 was significantly ($p < 0.05$) lower than that at pCd 8.2 and pCd 7.5 and was 0.88 d^{-1} (standard error of 0.01), indicating Cd toxicity. Koukal et al. (2003) found a EC_{50} for rate of photosynthesis value of $270 \mu\text{g Cd L}^{-1}$ (pCd about 5.6) for the green algae *Pseudokirchneriella subcapitata* at a pH of 8.5.

Total dissolved Cd concentrations increased significantly during the three days test by 10-20% of initial values in all treatments without synthetic ligands. This increase is probably the result of exudates produced by the algae that mobilize Cd from the resin. No significant changes in total dissolved Cd were found in the solutions where synthetic ligands were applied, likely as a result of the high $\text{CdL}:\text{Cd}^{2+}$ ratio. Koukal et al. (2007) assessed the formation of exudates by algae. The amount of exudates formed after three days and an initial cell density of $10^4 \text{ cells mL}^{-1}$ was equivalent to $0.25 \text{ mg DOC L}^{-1}$. Grassi and Mingazzini (2001) showed that the exudates formed by *Pseudokirchneriella subcapitata* are able to bind Cd^{2+} .

The uptake of Cd by the green algae increased with increasing Cd^{2+} activity in solution, however effects of ligands were small (Figure 2). Statistical analysis showed that uptake was unaffected by ligands at any pCd value ($p > 0.05$) except at the lowest ion activity (pCd 8.2) in the presence of NTA and CDTA. Here, Cd uptake flux was 2.2 times (NTA) and 2.8 times (CDTA) larger than that in the ligand free control. The total dissolved Cd in these treatments were factor 22 (NTA) and 35 (CDTA) larger than in the ligand free control, i.e. Cd uptake is more related to the free Cd^{2+} concentration than to total dissolved Cd, in line with the FIAM (Figure 2). The contribution of the complexes to Cd uptake, relative to that of the free ion, can be calculated from the Cd uptake in the ligand treatment (F_{uptake}) versus that in the ligand free treatment at same free Cd^{2+} activity ($F_{\text{uptake}}^{\text{contr}}$) along the same lines as for DGT:

$$\xi = \left(\frac{F_{\text{uptake}}}{F_{\text{uptake}}^{\text{contr}}} - 1 \right) \cdot \frac{[\text{Cd}^{2+}]}{[\text{CdL}]} \quad (5)$$

where $[\text{CdL}]$ and $[\text{Cd}^{2+}]$ are the bulk concentrations of the Cd complex and free ion, respectively. A ξ value of 1 implies that the complex contributes to the same extent as the free ion to uptake, in which case the uptake is proportional to the total metal concentration. A ξ value of 0 implies that the complex does not contribute and that the uptake is proportional to the free ion concentration, consistent with the FIAM. The contribution of NTA was 0.027 and that of CDTA was 0.052 at pCd=8.2.

The DGT measurements indicated that the Cd complexes with these synthetic ligands are able to contribute to the diffusion flux, and that their contribution to the diffusion flux follows the order: $\text{NTA} > \text{HEDTA} > \text{EDTA} \sim \text{EGTA} > \text{CDTA}$. Here, the contribution of the complexes at the lowest free Cd^{2+} activity to the Cd uptake by algae was: $\text{CDTA} > \text{NTA}$.

4 Discussion

Solution analysis during growth and across treatments suggests that pCd values were maintained despite contrasting total dissolved Cd concentrations, a set-up that allows to sensitively test the validity of the FIAM. Our results showed that Cd uptake, at free Cd²⁺ concentrations between 6 nM and 2 µM, by this green algae was hardly affected by the presence of synthetic ligands, in line with the FIAM. No effect of the synthetic ligands on the Cd uptake was observed at the two highest Cd²⁺ activities in this experiment. The validity of the FIAM at higher Cd concentrations was expected because of the relatively lower uptake rate per unit solution activity at higher metal concentrations than at lower metal concentrations. The Cd taken up by the algae relative to the Cd in solution decreases with increasing total dissolved Cd concentration. Only Cd complexes formed with NTA and CDTA at the lowest free Cd²⁺ activity were bioavailable, in contrast with the FIAM. However, these conditions in which Cd complexes exceed the free Cd²⁺ by a factor 35 or more are rarely found in freshwater systems, except in very soft, high DOC waters (Bradac et al., 2009; Baken et al., 2011).

The adherence of the Cd uptake to FIAM for unicellular algae was demonstrated previously for algae (Vigneault and Campbell, 2005), but not for periphyton (Meylan et al., 2004; Bradac et al., 2009), plants (Degryse et al., 2006 a,b) and the Caco-2 cells (Verheyen et al. 2012b). Vigneault and Campbell (2005) observed that the Cd uptake by *Pseudokirchneriella subcapitata* (Korshikov) Hindak and *Chlamydomonas reinhardtii* Dang at free concentrations between 1.5-14 nM, was controlled by the free Cd²⁺ concentration, not by the ligands. However, that study did not use a ligand free control study and it used a short time experiment in which metal sorption on the cell wall of the algae is a considerable fraction of Cd uptake. Our experiment was made for three days and during which algal growth and exudates are important. The effect of the changing

conditions on the metal uptake, i.e. the validity of FIAM, was not investigated before. Bradac et al. (2009) observed a contribution of Cd-NTA complexes to the Cd uptake by periphyton at Cd^{2+} activities of about 20 and 40 nM. This contribution was attributed to the diffusion of labile Cd-NTA complexes over an unstirred layer towards the periphyton layer across the cell membrane. Diffusion limitation was induced in the unstirred layer between the periphyton layer and the flowing water. The unstirred layer is more explicit above or around a large uptake surface (periphyton) than around a small uptake surface (algae). Less diffusion limitation is expected when the algae are suspended in solution instead of forming a layer. Along the same lines, labile complexes clearly enhance Cd uptake by plants at Cd^{2+} activities <10 nM (Smolders and McLaughlin, 1996; Oporto et al. 2009) and by Caco-2 cells (Verheyen et al. 2012b). These effects were also attributed to the contribution of complexes to the diffusion of Cd^{2+} towards the cells (see introduction). For algae, Fortin and Campbell (2000) showed that Ag uptake by the algae *Chlamydomonas reinhardtii* increased in the presence of chloride complexes at a free Ag^+ concentration of 8 nM.

The uptake flux divided by the solution concentration, termed permeability (P), is the critical factor that determines the extent of depletion of Cd adjacent to a membrane for a given thickness of an unstirred layer. The maximal diffusive flux (F) of a free ion towards a flat surface is obtained when surface concentration is zero and this flux equals $D[\text{Cd}^{2+}]/\delta$ with the diffusion coefficient D circa $5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, $[\text{Cd}^{2+}]$ the free Cd^{2+} concentration in the bulk solution and δ the thickness of an unstirred layer of water, e.g. 0.1 mm for plant roots. When $P = F/[\text{Cd}^{2+}] > D/\delta$, uptake is controlled by diffusion whereas internalization (P) controls uptake when the reverse is true. In the former case, complexes are likely to contribute to uptake provided they are kinetically labile. The D/δ ratio for plant roots is in the order of $10^{-4} \text{ cm s}^{-1}$ and Cd uptake flux divided by

the Cd^{2+} concentration in ligand free solutions is also about $10^{-4} \text{ cm s}^{-1}$, indicating that the actual uptake flux approaches the maximal diffusion flux and suggesting that the diffusive transport limits the uptake (Degryse et al., 2006a). For algae, however, the estimated diffusive layer thickness is much smaller. In case of spherical diffusion, i.e. assuming that algae are spheres, the diffusive layer thickness corresponds to the radius of the sphere (r), which is in the order of micrometers, and the value of D/δ is, therefore, about $10^{-2} \text{ cm s}^{-1}$. For uptake of Ag in algae, the permeability in unbuffered solutions was estimated $P \sim 10^{-2} \text{ cm s}^{-1}$ (Fortin and Campbell, 2000) explaining why complexes may contribute as observed. In contrast, the permeability of Cd by algae in our study for unbuffered solutions is much smaller than that of Ag. The value of P can be derived from the data in Fig.2 and is about $2 \times 10^{-6} \text{ cm s}^{-1}$, consistent with the results of Vigneault and Campbell (2005). In other words, the rate of internalization is much smaller than the estimated maximal average diffusive flux to the cell, suggesting that there is no diffusion limitation and explaining why the FIAM generally holds. The small and inconsistent deviation of FIAM for NTA and CDTA may be related to a underestimation of P : the diffusion flux may be confined to a much smaller surface than the average cell surface because of heterogeneous localization of transporters. Such can be quantified as outlined by Wilkinson and Buffle (2004). It can be shown that the observed uptake flux in our experiments would fit the maximal diffusion flux if there were only two transporters per cell (transporter density of circa $5 \times 10^{-18} \text{ cm}^{-2}$), which is an unrealistically low number (details not shown). Along the same lines, D/δ might be overestimated in above-mentioned estimates because the cells are no spheres but rather horse-shoe shaped (e.g. rods) and δ may extend perhaps $>10 \text{ }\mu\text{m}$. Still, the discrepancy between P and D/δ is 4 orders of magnitude, unlikely to be affected by the assumption. Thus, even taking into the uncertainties, the maximal diffusive flux remains substantially higher than the observed

uptake flux, which does support the hypothesis that the uptake is not transport limited. Other observations are also suggesting that diffusive transport is not limiting Cd uptake in algae. First, a clear competition effect between Cd^{2+} and Ca^{2+} was observed for Cd uptake in algae from unbuffered solutions (François et al., 2007; own unpublished results), suggesting that internalization, and not transport, is limiting the uptake. No such ion competition effects are found for Cd uptake in plants (e.g. pH effects) in chelator free solutions where transport, and not internalization controls uptake (Degryse and Smolders, 2012). Only at very high concentrations of labile complexes, sufficient to overcome the diffusion limitations, competition effects were found (Degryse et al., 2012). Second, whereas the contribution of the Cd complexes to the plant uptake followed the order of their dissociation rate (Degryse et al., 2006a), in line with their effect on transport, this is not as clearly the case for the uptake by algae here. Another reason for the contribution of complexes might be direct uptake. However, given that the complexes with the synthetic ligands are large hydrophylic complexes, this does not seem likely, and it also would not explain why there is no contribution of the complexes at large Cd^{2+} activities (Jackson and Morgan, 1978).

Concluding, Cd uptake in algae generally obeys the FIAM in the algal bottle test in which Cd^{2+} is buffered by a resin. Small exceptions were observed at the lowest Cd^{2+} activity where uptake increased 2-3 fold at Cd-L: Cd^{2+} ratio's 20-30. The uptake flux per unit free Cd^{2+} concentration, i.e. the permeability, is sufficiently low relative to the large potential flux towards the small cells and avoids considerable concentration gradients around the cells. Therefore, Cd complexes do not largely enhance the Cd bioavailability. Increasing cell growth rate or changing the ionic composition (higher pH, lower hardness), may increase the permeability to the point where the flux may not be sufficient, however such needs further assessments.

Acknowledgments

This work is financially supported by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) and was funded by the Onderzoeksfonds K.U.Leuven under the project number OT/08/020.

References

- Anderson, M.A., Morel, F.M.M., Guillard, R.R.L., 1978. Growth limitation of a coastal diatom by low zinc ion activity. *Nature*. 276, 70-71
- Bell, P.F., Chaney, R.L., Angle, J.S., 1991. Free metal activity and total metal concentrations as indices of micronutrient availability to barley. *Plant Soil*. 130, 51-62. *Hordeum-vulgare* (L.) 'Klages'
- Baken, S., Degryse, F., Verheyen, L., Merckx, R., Smolders E., 2011. Metal complexation properties of freshwater dissolved organic matter are explained by its aromaticity and by anthropogenic ligands. *Environ. Sci. Technol.* 45, 2584-2590.
- Bradac, P., Behra R., Sigg, L., 2009. Accumulation of Cd in periphyton under various freshwater speciation conditions. *Environ. Sci. Technol.* 43, 7291-7296.
- Degryse, F., Smolders, E., Merckx, R., 2006a. Labile Cd complexes increase Cd availability to plants. *Environ. Sci. Technol.* 40, 830-836.
- Degryse, F., Smolders, E., Parker, D.R., 2006b. Metal complexes increase uptake of Zn and Cu by plants: implication for uptake and deficiency studies in chelator-buffered solutions. *Plant soil*. 289, 171-185.
- Degryse, F., Smolders,E., Parker, D.R., 2006c. An agar gel technique demonstrates diffusion limitations to cadmium uptake by higher plants. *Environ. Chem.* 3, 419-423.

- Degryse, F., Smolders, E., 2012. Cadmium and nickel uptake by tomato spinach seedlings: plant or transport control? *Environ. Chem.* 9, 48-54.
- De Schamphelaere, K.A.C., Janssen, C.R., 2006. Bioavailability models for predicting copper toxicity to freshwater green microalgae as a function of water chemistry. *Environ. Sci. Technol.* 40, 4514-4522.
- Fortin, C., Campbell, P.G.C., 2000. Silver uptake by the green alga *Chlamydomonas reinhardtii* in relation to chemical speciation: influence of chloride. *Environ. Toxicol. Chem.* 19, 2769-2778.
- François, L., Fortin, C., Campbell, P.G.C., 2007. pH modulates transport rates of manganese and cadmium in the green alga *Chlamydomonas reinhardtii* through non-competitive interactions: Implications for an algal BLM. *Aquat. Toxicol.* 84, 123-132.
- Grassi, M., Mingazzini, M., 2001. ^{113}Cd -NMR and fluorescence studies of the interactions between Cd(II) and extracellular organic matter released by *Selenastrum capricornutum*. *Environ. Sci. Technol.* 130, 4271-4276.
- Jackson, G.A., Morgan, J.J., 1978. Trace metal-chelator interactions and phytoplankton growth in seawater media: theoretical analysis and comparison with reported observations. *Limnol. Oceanogr.* 23, 268-282.
- Jansen, S., Blust, R., Van Leeuwen, H.P., 2002. Metal speciation dynamics and bioavailability: Zn(II) and Cd(II) uptake by mussel (*Mytilus edulis*) and carp (*Cyprinus carpio*). *Environ. Sci. Technol.* 36, 2164-2170.
- Koukal, B., Guéguen, C., Pardos, M., Dominik, J., 2003. Influence of humic substances on the toxic effects of cadmium and zinc to the green alga *Pseudokirchneriella subcapitata*. *Chemosphere.* 53, 953-961.

- Koukal, B., Rossé, P., Reinhardt, A., Ferrari, B., Wilkinson, K.J., Loizeau, J., Dominik, J., 2007. Effect of *Pseudokirchneriella subcapitata* (Chlorophyceae) exudates on metal toxicity and colloid aggregation. *Water Res.* 41, 63-70.
- Lamelas, C., Slaveykova, V.L., 2007. Comparison of Cd(II), Cu(II), and Pb(II) biouptake by green algae in the presence of humic acid. *Environ. Sci. Technol.* 41, 4172-4178.
- Meylan, S., Behra, R., Sigg, L., 2004. Influence of metal speciation in natural freshwater on bioaccumulation of copper and zinc in periphyton: A microcosm study. *Environ. Sci. Technol.* 38, 3104-3111.
- OECD Test Guideline 201. 2006. Alga Growth Inhibition Test. In OECD Guideline for the testing of chemicals; OECD Publications Office: Paris, France.
- Oporto, C., Smolders, E., Degryse, F., Verheyen, L., Vandecasteele, C., 2009. DGT-measured fluxes explain the chloride-enhanced cadmium uptake by plants at low but not at high Cd supply. *Plant soil.* 318, 127-135.
- Parker, D.R., Norvell, W.A., Chaney, R.L., 1995. GEOCHEM-PC a chemical speciation program for IBM and compatible personal computers. In Chemical equilibrium and reaction models; Loeppert, R.H., Schwab, A.P., Goldberg, S., Eds., SSSA special publication. 42: Madison, WI.
- Schaider, L.A., Parker, D.R., Sedlak, D.L., 2006. Uptake of EDTA-complexed Pb, Cd and Fe by solution- and sand-cultured *Brassica juncea*. *Plant soil.* 286, 377-391.
- Santore, R.C., Mathew, R., Paquin P.R., Di Toro, D., 2002. Application of the biotic ligand model to predicting zinc toxicity to rainbow trout, fathed minnow, and *Daphnia magna*. *Comp. Biochem. Phys. Part C.* 133, 271-285.
- Smolders, E., McLaughlin, M.J., 1996. Chloride increases cadmium uptake in Swiss chard in a resin-buffered nutrient solution. *Soil Sci. Soc. Am J.* 60, 1443-1447.

Verheyen, L., Merckx, R., Smolders, E. 2012a. A resin-buffered nutrient solution for controlling metal speciation in the algal bottle assay. *Aquatic toxicology*. DOI: 10.1016/j.aquatox.2012.02.021

Verheyen, L., Degryse, F., Niewold, T., Smolders, E. 2012b. Labile complexes facilitate cadmium uptake by Caco-2 cells. *Science of the total environment*. DOI: 10.1016/j.scitotenv.2012.03.044

Vigneault, B., Campbell, P.G.C., 2005. Uptake of cadmium by freshwater green algae: effects of pH and aquatic humic substances. *J. Phycol.* 41, 55-61.

Wilkinson, K.J., Buffle, J., 2004. *Physicochemical Kinetics and Transport at Biointerfaces*, John Wiley & Sons, chapter 10: Critical evaluation of physicochemical parameters and processes for modelling the biological uptake of trace metals in environmental (aquatic) systems, pp. 509-510.

Zhang, H., Davison, W., 2000. Direct in situ measurements of labile inorganic and organically bound metal species in synthetic solutions and natural waters using diffusive gradients in thin films. *Anal. Chem.* 72, 4447-4457.

5 Table

Table 1. The nominal ligand concentrations (μM) and measured initial total dissolved Cd (μM , standard deviations in brackets, $n=2$) in the initial uptake solutions containing three different Cd^{2+} activities (pCd 8.2; pCd 7.5; pCd 5.7) buffered with a chelating resin. The concentrations of the synthetic ligands were selected to obtain not only a constant free Cd^{2+} activity (buffered by the resin during the experiment) but also a similar total Cd concentration among the treatments with different ligands. The required concentrations were estimated by GEOCHEM-PC

Ligand	pCd 8.2		pCd 7.5		pCd 5.7	
	ligand	Cd	ligand	Cd	ligand	Cd
control	-	<0.02	-	0.03	-	2.2
NTA	30	0.13(<0.01)	30	1.10(<0.01)	40	23.0 (0.3)
HEDTA	0.49	0.07(<0.01)	1.0	0.44(<0.01)	9.7	8.6(0.1)
EDTA	0.51	0.12(<0.01)	1.0	0.53(0.03)	10	9.4(<0.1)
CDTA	0.40	0.21(0.01)	1.0	0.80(<0.03)	9.9	11.0(<0.1)
EGTA	0.29	0.12(<0.01)	1.0	0.71(0.04)	10	11.0(<0.1)

6 FIGURES

Figure 1 Relationship between the DGT labile species (c_{DGT}) and the total dissolved Cd concentration (Cd_{tot}) in solutions containing complexes with synthetic ligands. All solutions had a pCd of 8.2. The full line is the 1:1 line which is valid for ligand free solutions

Figure 2 The surface based uptake flux (3 days exposure) as a function of the free Cd^{2+} activity (top) or the total dissolved Cd concentration (bottom) in solutions without complexes (no ligand), or in solutions with complexes (ligand specified in legend) at a concentration of circa 20-fold the free ion concentration for the two lowest series and at a concentration of circa 5-fold the free ion concentration for the highest series. Standard error bars are given but are smaller than the symbols for some treatments.

Figure 1

Figure 2