Cadmium thiosulfate complexes can be assimilated by a green alga via a sulfate transporter but do not increase Cd toxicity

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Abstract

Rationale. For a given free metal ion activity in the exposure solution, the Biotic Ligand Model assumes that metal uptake will be independent of the various ligands present in solution that are buffering [M^{z+}]. In this context, we have evaluated cadmium bioavailability in the absence or presence of thiosulfate, using *Chlamydomonas reinhardtii* as the test alga.

Methodology. Short-term exposures (≤ 41 min) were run with a fixed concentration of the free Cd^{2+} ion (3.0 ± 0.1 nM), buffered with either nitrilotriacetate or thiosulfate, to determine Cd uptake. Subsequent long-term exposures (72 h) over a range of free Cd^{2+} concentrations were used to determine the effects of Cd on algal growth.

Results. Contrary to Biotic Ligand Model predictions, Cd uptake was enhanced when Cd^{2+} was buffered with thiosulfate. Removal of sulfate from this exposure medium increased Cd uptake; conversely, if [SO_4] was increased, Cd uptake decreased. In the absence of thiosulfate, Cd uptake was unaffected by changes in [SO_4]. In the long-term exposures, the cellular Cd quota needed to reduce algal growth by 50% was significantly higher in the presence of thiosulfate than in its absence.

Discussion. In the presence of thiosulfate, Cd can enter the algal cell not only by cation transport but also by transport of the intact Cd-thiosulfate complex via the anion transporter responsible for sulfate uptake. We speculate that some of the Cd taken up by anion transport remains in complexed form and is less bioavailable than the Cd that enters the cell via cation transport.

Key words

anionic uptake, cationic uptake, Cd, cell quota, growth inhibition, intracellular metal speciation, phytoplankton, sulfate, thiosulfate, trace metals, unicellular alga.

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Environmental context

Thiosulfate is present in natural waters, especially those influenced by sulfide oxidation, and it has a marked affinity for metals such as cadmium. Normally the binding of cadmium by thiosulfate would be expected to reduce the metal's bioavailability. However, here we demonstrate that algal uptake of cadmium is enhanced in the presence of thiosulfate, indicating that Cd can enter the alga via a novel route as an intact Cd-thiosulfate complex.

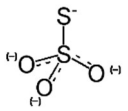
Introduction

Over the past 30+ years, studies of metal-alga interactions have contributed greatly to our understanding of the mechanisms of metal movement across biological membranes. In the great majority of experiments carried out with appropriate control of metal speciation in the exposure medium, the best predictor of algal metal uptake and toxicity has proven to be the free metal ion concentration. Early demonstrations of this dependence on $[M^{z+}]$ led to the formulation of the Free Ion Activity Model (FIAM) (Morel 1983), which has evolved into what is now referred to as the Biotic Ligand Model or BLM (Campbell et al. 2002).

This dependence of metal uptake and toxicity on the free metal ion activity makes biophysical sense, given that the vast majority of metal forms existing in aqueous solution are charged and hydrophilic, and consequently cannot traverse biological membranes by simple diffusion. Instead, metals must normally cross biological membranes by facilitated transport, which in the case of divalent metals typically involves the binding of the free metal ion to a carrier protein present in the cell membrane (Blaby-Haas and Merchant 2012).

There are a number of recognized exceptions to this generalization, including the uptake of lipophilic metal complexes by simple diffusion (e.g., lipophilic $Cd(L)_2^0$ complexes with diethyldithiocarbamate and with ethylxanthate – Boullemant et al. (2009)) and the uptake of hydrophilic metal-ligand complexes (Campbell et al. 2002, Campbell and Fortin 2013). In this latter case, the ligand itself must be able to cross the biological membrane by facilitated transport, and the membrane transporter must be sufficiently undiscriminating as to accommodate both the free ligand and the metal-ligand complex. Examples of such ligands include both organic molecules (e.g., citrate and histidine (Errécalde and Campbell 2000, Chivers et al. 2012)) and inorganic anions (e.g., thiosulfate (Fortin and Campbell 2001)).

In the present study we have focused on thiosulfate, a partially oxidized sulfur anion that is readily assimilated by algae (Biedlingmaier and Schmidt 1989) and that forms strong inner-sphere complexes with "soft" cations such as Ag, Cd and Hg. Note that although the CdS_2O_3 complex appears to be uncharged, in fact the Cd ion retains a charge of +1 and the oxygen atoms of the thiosulfate ligand retain a delocalized charge of -1. Thiosulfate can be found in natural waters at the interface between oxic and anoxic environments (Kondo et al. 2000), in some industrial effluents (Purcell and Peters 1998), in ponds used to treat tailings from copper, lead, nickel and zinc mines (Lu and Wang 2012), and in hydrothermal waters (Druschel et al. 2003). In such environments, thiosulfate concentrations approaching 1 mM have been reported.



In earlier work we demonstrated that thiosulfate greatly facilitated the uptake of silver by a unicellular alga, *Chlamydomonas reinhardtii* (Fortin and Campbell 2001), and that this enhancement was favoured when the concentration of sulfate in the exposure medium was low. In a subsequent study, designed to determine the influence of thiosulfate on the toxicity of silver, we demonstrated that the enhanced uptake of silver in the presence of thiosulfate did not lead to a proportional increase in toxicity, suggesting that the silver-thiosulfate complex did not fully dissociate when it entered the intracellular environment (Hiriart-Baer et al. 2006).

The objective of the present study was to determine how thiosulfate influences the uptake and toxicity of Cd, another metal that forms reasonably stable thiosulfate complexes. To that end, we performed short-term uptake experiments with *C. reinhardtii*, in which the free Cd^{2+} concentration was held constant in the absence or presence of thiosulfate. We then carried out long-term exposures (72 h), over a range of free Cd^{2+} concentrations and in the absence or presence of thiosulfate, to determine if the enhanced uptake of Cd in the presence of thiosulfate led to increased toxicity.

The unambiguous interpretation of such experiments is only possible if the speciation of the studied metal Is known (Twiss et al. 2001). One of the advantages of using unicellular algae for such experiments is that they can be grown and exposed in defined media, greatly facilitating the use of chemical equilibrium modelling software to calculate metal speciation. However, if the exposure period is prolonged, the presence in the medium of algal exudates of unknown composition may compromise such calculations. In such cases, it is essential to be able to actually measure the free cation concentration in the exposure medium (Batley et al. 2004). In the present experiments, when the exposure periods were prolonged to allow determination of algal growth rates and yields, we used an

ion exchange technique to determine how the free Cd^{2+} concentration evolved over the course of the exposure.

Methods

Reagents and labware

All plasticware was soaked for at least 24 h in 10% HNO_3, thoroughly rinsed seven times with ultrapure water (18 MΩ·cm) and dried under a laminar flow hood prior to use. Polycarbonate flasks were used for the metal exposure experiments. Salts used for cultures and experiments were of analytical grade or better. Radioactive cadmium (^{109}Cd 376 - 81085 mCi·mmol^{-1}) was purchased from Amersham Canada (Oakville, ON, Canada). The radioactivity of ^{109}Cd was determined with a Wallac 1480 Wizard gamma counter (Perkin Elmer Life Sciences, Turku, Finland); counts between 16 – 36 keV were considered for ^{109}Cd. Acidic stock solutions of cold and radioactive Cd were kept at pH < 2 in the dark at 4°C.

Organism and culture conditions.

The experiments were carried out with *Chlamydomonas reinhardtii*, a euryhaline unicellular green alga obtained from the University of Toronto Culture Collection (UTCC11). The alga was grown axenically in modified high salt medium, MHSM (modified from Macfie et al. (1994); see Table S1, Supplementary Material) with an ionic strength of 6 meq·L^{-1}. The growth medium was buffered at pH 7 by the addition of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; 10 mM). To avoid metal precipitation, culture media were sterilized by autoclaving before the addition of the trace micronutrient mix. This latter solution was sterilized by filtration through a 0.2 µm polycarbonate membrane (Millipore) before addition into the growth medium. Axenic batch cultures of 100 mL were maintained under constant illumination at 100 ± 10 µE·m^{-2}·s^{-1} (Cool White Fluorescent Tubes), with rotary agitation at 50 rpm and a temperature of 20°C in 250 mL polycarbonate Erlenmeyer flasks. For regular maintenance, ~2 mL of culture was transferred to a fresh, sterile medium (pH = 7) every week. Cultures were periodically checked for bacterial contamination by plating onto nutrient agar (Difco-Bacto agar). All manipulations of the algal cultures were carried out within a laminar flow hood with sterile materials.

Short-term metal exposure experiments

Short exposure periods (< 1 h) were used for the determinations of cadmium uptake, to minimize the influence of the algae on their exposure medium. These uptake experiments were designed to expose the algal cells to a fixed low free metal concentration [Cd^{2+}] of 3.0 ± 0.1 nM, in the

absence or presence (1.0 mM) of thiosulfate. The sulfate concentration was also varied (0-400 µM), since our previous experiments with silver and *C. reinhardtii* had shown that sulfate could compete with thiosulfate for uptake via the same anion transporter (Fortin and Campbell 2001). When sulfate was removed or added in excess to the exposure solution, the nitrate concentration was adjusted so as to maintain a constant ionic strength of 6 meq·L^{-1} in the medium. The general procedure involved the following steps.

i. Exposure solutions were prepared with the basic MHSM ingredients, without the micronutrients, but with either nitrilotriacetate (NTA) or thiosulfate added to buffer the Cd concentration; the compositions of these simplified exposure solutions are summarized in Table 1. The stock solutions used to prepare the exposure media were pre-filtered (0.2 µm, polycarbonate filter). The exposure solutions were allowed to equilibrate for 24 h before the inoculation step (iii). The known composition of the simplified exposure solution was used to calculate the speciation of the radioisotope, using chemical equilibrium software (MINEQL 5; Schecher and McAvoy (2001) – see *Metal speciation*, below).

ii. Exponentially growing algal cells were removed from a *Chlamydomonas reinhardtii* culture, collected by centrifugation and rinsed thoroughly to remove all traces of the original growth medium. The rinse solutions corresponded to the MHSM solution but without the macro- and micronutrients; the composition of the rinse solutions can be found in the Supplementary Information, Table S1.

iii. After the final rinse, the algal cells were resuspended in a known volume of the simplified rinse solution and a small volume was removed to determine the number of algal cells and their surface area (Beckman Coulter Counter Multisizer III; dilution of 0.1 mL of the suspension in 9.9 mL of an isotonic solution, Isoton® III). Known volumes of the suspension were then used to inoculate the algae into the Erlenmeyer flasks containing the exposure medium + the radioisotope, as prepared in step (i). Target values for initial cell counts were 30,000 cells·mL^{-1}.

iv. For a given short-term exposure condition, three replicate flasks were incubated under the same conditions of light and temperature as described for the algal maintenance cultures (i.e., 100 ± 10 µE·m^{-2}·s^{-1}, rotary agitation at 50 rpm, 20°C).

v. In the experiments designed to follow Cd uptake kinetics, subsamples were removed after 11, 20, 31 and 41 min. In the experiments performed to test the influence of the sulfate

concentration on Cd uptake, samples were collected after a single fixed period (30 min). In both cases, the algal cells were collected by filtration onto two superimposed polycarbonate filters (Millipore) and rinsed with a solution containing ethylenediamine-tetraacetate to remove the adsorbed metals ([EDTA] = 0.1 mM – Hassler et al. (2004)). The two filters were placed in separate scintillation vials (5 mL) containing 2 mL of ultrapure water, and their radioactivity measured. The activity of the lower filter was used to correct that of the upper filter for any retention of the radioisotope by the filter membrane itself. Counting time was set at 2000 s or to a maximum collection of 100,000 counts in order to keep analytical errors below 5%. Counts per minute (cpm) were converted into molar concentrations of the radioisotope, taking into account the detector efficiency (40.0%), the specific activity specified by the supplier, and radioactive decay. Note that Cd uptake refers to intracellular Cd and is normalized with respect to the algal density, expressed in surface area (i.e., nmol $Cd \cdot m^{-2}$).

Longer-term growth inhibition experiments

As a complement to the short-term Cd uptake experiments, we also carried out growth inhibition experiments with Cd (up to 72 h) and separate Cd uptake experiments (also over a 72-h period). The exposure media for these experiments were based on the whole MHSM, including the macro- and micronutrients (see Tables S2 and S3). For the inoculation, algal cells were harvested as for the uptake experiments, rinsed, and introduced into the growth medium buffered with 15 µM EDTA (either alone or jointly with thiosulfate, 1 mM). The free Cd^{2+} concentration was calculated with MINEQL+ and it was checked using an Equilibrium Ion Exchange technique (IET; Fortin and Campbell (1998) – see *Metal speciation*, below). Algal growth was followed over 72 h, under the same conditions of light and temperature as those described for the algal maintenance cultures. Cell numbers were determined on subsamples (1 mL) that were removed from the Erlenmeyer flasks at regular time intervals (t = 0, 3.5, 24, 32, 48 and 72 h for the growth inhibition studies; t = 6, 12, 24, 36, 48 and 72 h for the long-term uptake experiments).

The growth inhibition studies were performed at two separate times, to test the reproducibility of the results; in the following text, these are referred to as series A and B. In each series, we ran two simultaneous experiments, one without thiosulfate in order to obtain a reference EC50 value for *C. reinhardtii*, and one with thiosulfate. To facilitate the comparison of the results of the different experiments, we exposed the cells to a similar range of Cd^{2+} concentrations in the experiments with or without thiosulfate. In these media, the Cd was well buffered (< 6% of the Cd in the form of free Cd^{2+})

(Tables S4 and S5). The aim here was to keep the Cd^{2+}/Cd_T ratio as low as possible, to maintain stable exposure conditions during the 72-h exposure period (e.g., to avoid potential metal depletion over time due to metal assimilation or adsorption).

For these experiments, we had three replicates with eight different exposure media: one control medium (MHSM), one medium with the ligands added (i.e., EDTA + thiosulfate but no Cd), and six media with increasing [Cd^{2+}] (3 nM → 1 µM) – see Tables S4 and S5. To determine the toxicity of cadmium in the presence or absence of thiosulfate, we then monitored algae growth rate (µ) and yield (Y) over 72 h. Growth rates were calculated for the periods 3.5-24 h, 24-29 h and 29-48 h, using the cell numbers determined at the beginning and end of each period. The final yields were determined on the basis of the cell numbers attained after 72 h. In similar long-term experiments, we also tracked the accumulation of Cd in the cells at each time step, using the same filtration and rinsing procedure that was used for the short-term exposures.

Metal speciation

Metal speciation calculations were performed using MINEQL+ software (Schecher and McAvoy 2001). The thermodynamic constants used for interactions of Cd^{2+} and H^+ with the three principal ligands used in these experiments (i.e., thiosulfate, NTA and EDTA) are those of Martell et al. (2004) and are compiled in the Supplementary Information, Table S6.

Metal speciation measurements were performed only in the long-term (72-h) experiments with Cd, where we anticipated that algal exudates might accumulate and bind some of the Cd (Paquet et al. 2015). The free Cd^{2+} concentration was determined using an ion-exchange technique (IET), as described in Fortin and Campbell (1998). This method involved equilibrating a sulfonic acid type cation exchange resin (Dowex 50W-X8) with a series of MHSM solutions to which had been added known amounts of Cd. After equilibration, the column was eluted with concentrated nitric acid. By measuring the amount of Cd retained by the column for a given free Cd^{2+} concentration (as calculated for the MHSM solutions using MINEQL+) we could establish a conditional equilibrium constant for Cd binding to the resin. The calibrated resin was then used to determine the free Cd^{2+} concentration in samples collected from the algal growth media at t = 6, 12, 24, 36, 48 and 72 h. Additional details about the IET approach can be found in the Supplementary Material.

Statistics

To compare and determine if there was a significant difference between the points from experiments with or without thiosulfate in the exposure media, we carried out a one-way analysis of

variance (ANOVA) using SYSTAT software (SPSS *Science* Marketing Department, Chicago, IL, USA; version 10). This type of test requires normality of the data (Kolmogorov-Smirnov test) and homogeneity of the variances. If these conditions were met, the *a posteriori* SNK (Student-Newman-Keuls) test was used to determine which points were significantly different ($P < 0.05$).

For the growth inhibition experiments, effective concentration values (EC50s) were determined using the US Environmental Protection Agency's Toxicity Relationship Analysis Program (Version 1.30a) by nonlinear regression. The logistic equation with two parameters was used.

Results

Short-term experiments

The short-term uptake experiments were all performed with a free Cd^{2+} concentration of 3.0 ± 0.1 nM. Uptake over the first 40 min increased linearly and it was enhanced in the presence of thiosulfate (Figure 1). Comparison of Cd uptake in the absence and presence of thiosulfate suggests that about 50% of the Cd accumulated after 41 min cannot be explained by uptake of the free Cd^{2+} cation. This enhancement was even greater in the medium where the sulfate had been removed (the upper plot in Figure 1). Short-term Cd uptake was unaffected by the sulfate concentration (up to 400 µM) when thiosulfate was absent (Figure 2). However, in a similar experiment where thiosulfate was present (1.0 mM), the addition of sulfate over the same range of concentrations did result in decreased Cd uptake (Figure 3).

Longer-term experiments

The longer-term manipulations involved exposing *C. reinhardtii* to Cd for 72 h and following (i) Cd concentrations and speciation in the exposure medium, (ii) Cd accumulation within the algal cells and (iii) algal growth over the whole exposure time. These experiments, carried out in triplicate in MHSM with its full complement of micro- and macronutrients, were designed to determine if the effect of thiosulfate on Cd accumulation persisted for the whole exposure period and, if so, if Cd inhibition of algal growth was also greater in the presence of thiosulfate.

Cd in the exposure media

Total dissolved Cd and free Cd^{2+} were determined in samples collected from the exposure flasks at t = 0, 6, 12, 24, 36, 48 and 72 h. As indicated in Figure 4, the relative concentration of free Cd^{2+} was stable during the first 12 hours in the presence or absence of thiosulfate. Subsequently, between 12 and 24 h, there was a decrease in free cadmium of about 30% in both types of media. After this drop, the free Cd^{2+} concentration remained stable (60-70% of the initial value), regardless of the exposure

medium. Throughout this period (from 0 to 72 h), the total dissolved Cd concentration remained substantially stable (decrease of ≤ 11%).

Cd accumulation in the algal cells

The initial free Cd^{2+} concentration (3 nM) in the growth medium was buffered only with EDTA (15 µM) or with a mixture of thiosulfate (1 mM) and EDTA (15 µM). As indicated in Figure 5, Cd accumulated by *C. reinhardtii* uptake was consistently greater throughout the exposure period when the medium contained thiosulfate. For the final time point, the intracellular Cd concentration was about 30% higher when the algae were grown in the presence of thiosulfate.

Growth inhibition

Despite the difference in intracellular Cd, growth rates calculated for the exponential phase (5 → 24 h), transitional phase (24-29 h) or late exponential phase (29-48 h) did not differ statistically between the media with or without thiosulfate. However, in plots of the final yield after 72 h as a function of the initial free Cd^{2+} concentration, the yields in the media containing thiosulfate were slightly higher than the yields in the media with EDTA alone (Figures S1 and S2; see Table 2 for EC50 values expressed as nM Cd^{2+}). Because the speciation of cadmium varied over the course of the experiment, with the variation being slightly different between the media with or without thiosulfate (as shown in Figure 4), we also plotted the final yield of algal cells after 72 h as a function of the Cd cell quota (fmol $Cd \cdot cell^{-1}$) (Figures 6A and 6B). These plots confirm that for a given significant reduction in cell yield, the Cd cell quotas were statistically higher in the presence of thiosulfate than in its absence (Table 2).

Discussion

Short-term experiments

Dual uptake routes for Cd

The results of the short-term Cd uptake experiments are compatible with our initial hypothesis, that Cd can enter *C. reinhardtii* both as a cation and as a thiosulfate complex. The algal cell membrane hosts both cation and anion transporters, with the latter known to be somewhat less selective than cation transporters (Stein 1990). In the present case, it seems reasonable to assume that three species are competing for transport with the sulfate anion transporter: sulfate, thiosulfate and the thiosulfate-Cd complex. Since the Cd^{2+} cation forms only weak, outer-sphere complexes with sulfate, we discount the possibility that $CdSO_4$ could enter the algal cell via the sulfate transporter. In other words, changes in sulfate concentration would not be expected to affect Cd uptake in the absence of thiosulfate and indeed this was what was observed (Figure 2) and confirmed by analysis of variance. However, when

thiosulfate was present at a constant concentration (1 mM), addition of sulfate did cause a decrease in the rate of Cd uptake, presumably reflecting increased competition between SO_4^{2-} and $S_2O_3^{2-}$ for transport by the anion transporter (Figure 7). A similar effect of sulfate was noted in our earlier work on the uptake of silver by *C. reinhardtii* in the presence of thiosulfate (Fortin and Campbell 2001). Note that several researchers have reported a similar competitive effect between sulfate and thiosulfate in sulfate uptake experiments conducted on unicellular algae (Biedlingmaier and Schmidt 1989, Pérez-Castineira et al. 1998). The present results, with Cd 'hitching a ride' into the algal cell, are an example of the role of molecular mimicry in the uptake of non-essential metals (Bridges and Zalups 2005).

Relative efficiency of the cation and anion uptake routes

The data shown in Figure 1 were used to provide a rough estimate of the relative efficiency (e.g., uptake rate of a Cd species/concentration of the Cd species) of the two different uptake mechanisms (i.e., through cation and anion transporters). Uptake by both routes would be expected to follow Michaelis-Menten type kinetics. The data generated in the present study are insufficient to perform such an analysis, but using Figure 1 we compared the rate of Cd uptake in the absence of thiosulfate with that in the presence of thiosulfate but without any sulfate (i.e., the lowest and highest rates of uptake in Figure 1). In both cases the concentration of free Cd^{2+} was 3.0 nM, meaning the difference between the slopes of the two plots can be attributed to uptake of the Cd thiosulfate complexes. This simple calculation shows that the two uptake routes made roughly equal contributions to the total Cd accumulation. However, the concentration of the CdS_2O_3 complex (11.6 nM) was approximated 4 times higher than the concentration of free Cd^{2+}. If we assume a linear relationship between uptake of the complex and its concentration (i.e., that the complex is present at a concentration well below the K_m value for the sulfate transporter), then the relative "efficiency" of the anion transporter would be about ¼ that of the cation transporter.

The hypothesis of diffusional control of Cd uptake can be rejected

Species other than the free metal cation can contribute to metal uptake by unicellular algae when the rate of metal uptake is controlled not by the movement of the metal across the algal membrane, but rather by diffusion of the metal from the bulk solution to the algal surface, i.e., through the boundary layer surrounding the algal cell (Buffle et al. 2009). In the short-term uptake experiments, we kept the free Cd^{2+} concentration constant but manipulated Cd speciation. In the experiments run in the presence of thiosulfate, the total Cd concentration was higher than in the experiments run without thiosulfate (see Table 1). If the accumulation of cadmium by *C. reinhardtii* were controlled by the

transport of labile Cd species from the bulk solution to the algal surface, a higher accumulation in the algae could be explained by the diffusion and dissociation of the labile Cd-thiosulfate complex even if only the free Cd^{2+} were internalized. Such cases are rare but several have been documented, notably for Ag^{1+} and *C. reinhardtii* (Fortin and Campbell 2000). In the present case, however, this hypothesis is inconsistent with the observation that increasing the sulfate concentration reduces Cd uptake in the presence of thiosulfate (Figure 3) but not in its absence (Figure 2). The effect of sulfate can only be explained if the sulfate transporter is involved in Cd uptake when thiosulfate is present.

As further confirmation that the rate-limiting step in Cd accumulation by *C. reinhardtii* was not diffusion of Cd from the bulk solution to the algal surface, we calculated the uptake flux of Cd and compared it to the predicted maximum flux of free Cd^{2+} across the boundary layer. To do so we used the maximum uptake rate (i.e., Figure 1, plot with thiosulfate but no sulfate), expressed as pmol $Cd \cdot m^{-2} \cdot s^{-1}$ (1.51×10^{-4} pmol $Cd \cdot cm^{-2} \cdot s^{-1}$), and then compared this rate with the diffusional flux of Cd across the unstirred boundary layer, assuming a boundary layer thickness of 8×10^{-4} cm. The calculated algal uptake flux was 1.51×10^{-4} pmol Cd $cm^{-2}s^{-1}$ whereas the diffusional flux was 1.31×10^{-1} pmol$\cdot cm^{-2}s^{-1}$. In other words, the computed diffusional flux for the free Cd^{2+} ion under these conditions is almost 1000 times higher than the algal uptake rate, meaning that diffusional limitation of Cd uptake cannot be invoked to explain our experimental results. The details of these calculations can be found in the Supplementary Information.

Longer-term experiments

Cadmium behaviour in the exposure solution

The algal growth experiments were performed to determine whether the increased uptake of Cd in the presence of thiosulfate would lead to increased toxicity (i.e., reduced growth). Over the course of the 72-h exposure period, losses of total dissolved Cd from the growth media were less than 11% (largely due to algal uptake). In such batch experiments with metals, there is always some incertitude about the speciation of the metal, given that algal exudates with functional groups capable of binding Cd may potentially accumulate in the growth medium.

To maintain reasonably stable exposure conditions in the growth media, we buffered the free Cd^{2+} concentration with EDTA (15 µM) such that < 6% of the total Cd was present as free Cd^{2+}. The free Cd^{2+} concentration was practically stable during the first 12 h of exposure (Figure 4) and it was close to the theoretical Cd speciation in the exposure media, as calculated with MINEQL+ software (Tables S7 and S8). This initial period corresponds to the lag phase, followed by the start of exponential growth.

Algal growth is slow and the cells (relatively few in number) have little influence on the exposure medium. Subsequently, between 12 and 24 h, the free Cd^{2+} concentration decreased by approximately 35% in the two media; during this period the algal cells presumably began to respond to cadmium exposure. After the first day of exposure, the concentration of free cadmium remained stable at ~65% of the initial concentration, regardless of the exposure medium. In this period the proportion of free Cd^{2+} was slightly lower in the growth media containing both EDTA and thiosulfate, compared to those with EDTA alone (Figure 4), but this difference was not statistically significant.

The decrease in $[Cd^{2+}]$ between 12 and 24 h does not reflect a loss of Cd from solution but rather a change in its speciation. We suspect that this change in Cd speciation results from the excretion of ligand(s) by *C. reinhardtii*. To compete with EDTA and thiosulfate to complex Cd in solution and thus disrupt Cd speciation, the excreted ligand(s) must have a relatively high conditional complexation constant since the excreted concentrations are most likely low.

Algal response

The long-term experiments confirmed the results of the short-term experiments, in that they showed that algal accumulation of Cd was greater in the presence of thiosulfate over the whole 72-h period. However, examination of algal growth over this period did not show any evidence of greater toxicity of Cd in the presence of thiosulfate. Indeed, in the plots of final algal yield against the Cd cell quota (Figures 6A and 6B), we see the opposite response, namely that EC_{50} concentration (expressed as fmol Cd·cell^{-1}) is *higher* in the presence of thiosulfate than in its absence (Table 1). For a 50% reduction in yield, 1.5 times more Cd must be accumulated when thiosulfate is present in the exposure media. In other words, in the presence of thiosulfate, more Cd is accumulated within the algal cells but this intracellular Cd is less bioavailable (i.e., less toxic) than the Cd that is accumulated in the absence of thiosulfate. Clearly, the subcellular partitioning of Cd must be different in the two experiments. We speculate that this may be because some of the Cd that enters the cell as the thiosulfate complex remains in this form and is less bioavailable internally. Alternatively, perhaps the Cd that enters the cell with thiosulfate is handled differently within the cell and is detoxified more effectively than the Cd that enters the cell as Cd^{2+}. Finally, based on examination of Figures 6A and 6B, it would appear that a cellular quota of about 0.5 fmol·cell^{-1} is tolerated by *C. reinhardtii*, whether or not thiosulfate is present. Once this threshold is exceeded, the protective role of thiosulfate on the toxicity of intracellular cadmium comes into play.

Conclusions

The biotic ligand model, with its intrinsic link to the free metal ion concentration in the exposure medium, underestimates cadmium uptake in the presence of an assimilable ligand such as thiosulfate. There is clear evidence for uptake of the intact CdS_2O_3 complex, via a sulfate transporter (as shown earlier for silver ($AgS_2O_3^{-1}$) (Fortin and Campbell 2001, Hiriart-Baer et al. 2006)). Sulfate transporters are ubiquitous in freshwater algae and we suspect that the present results are not specific to *Chlamydomonas reinhardtii*. Low ambient sulfate concentrations would mean reduced competition with thiosulfate and also up-regulation of sulfate transport, both of which would favour uptake of thiosulfate and its complexes with Ag and Cd. Finally, it is clear from the present results with Cd and from the earlier results with Ag that total intracellular metal contents, i.e., cell metal quotas, are not unambiguous predictors of metal toxicity. Just as metal speciation in the exposure medium affects metal bioavailability, so too does metal speciation in the subcellular environment. The present results clearly suggest that the intracellular speciation of Cd should be probed in the absence and presence of thiosulfate.

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Conflicts of Interest

The authors declare no conflict of interest.

Data Availability

All raw data used to prepare the figures are publicly available at https://doi.org/10.5683/SP3/LWHVU9 [This link will be activated once the manuscript has been accepted for publication. In the meantime, reviewers can access the data file through this temporary private URL link: https://dataverse.scholarsportal.info/privateurl.xhtml?token=3763aeda-a074-412c-a677-b44c204a4d82]

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Tables

Table 1: Exposure conditions for the short-term uptake experiments (< 1 h). In addition to the variables shown in this table, all the exposure media contained the major MHSM constituents at the concentrations indicated in Table S1, column 3, lines 1 to 10.

Experiment description	$[Cd]_T$ (nM)	$[Cd^{2+}]$ (nM)	$[SO_4^{2-}]$ (μM)	$[S_2O_3^{2-}]$ (mM)	[NTA] (nM)
Figure 1, sulfate and NTA	8.65	3.0 ± 0.1	81.2	–	100
Figure 1, sulfate and thiosulfate	18.0	3.0 ± 0.1	81.2	1.0	–
Figure 1, thiosulfate alone	17.8	3.0 ± 0.1	–	1.0	–
Figure 2, variable sulfate, no thiosulfate	8.65	3.0 ± 0.1	0 – 400	–	100
Figure 3, variable sulfate, with thiosulfate	17.8	3.0 ± 0.1	0 – 400	1.0	–

Table 2: Effective concentration values (EC50) calculated from the relative yields obtained in the algal growth media without and with thiosulfate (Figures 6A and 6B). The values are expressed as cellular Cd quotas (femtomoles Cd·cell^{-1}) $\pm$ SD (n = 3) and as Cd^{2+} concentrations in the exposure media (nM Cd^{2+}) $\pm$ SD (n = 3).

	EC50 (fmol Cd·cell^{-1})		EC50 (nM Cd^{2+})	
	Series A	Series B	Series A	Series B
With thiosulfate	2.38 ± 0.16[(1)]	2.16 ± 0.25[(1)]	0.73 ± 0.11[(1)]	0.74 ± 0.19[(1)]
Without thiosulfate	1.77 ± 0.24[(1)]	1.35 ± 0.14[(1)]	0.41 ± 0.09[(1)]	0.25 ± 0.11[(1)]

[(1)] Significant difference ($P < 0.05$) between values in a given series. Series A and B were conducted at separate times, to test the reproducibility of the results. In each series, two simultaneous experiments were performed, one without thiosulfate in order to obtain a reference EC50 value for *C. reinhardtii*, and one with thiosulfate. Each such experiment was run with three replicates.

Figures

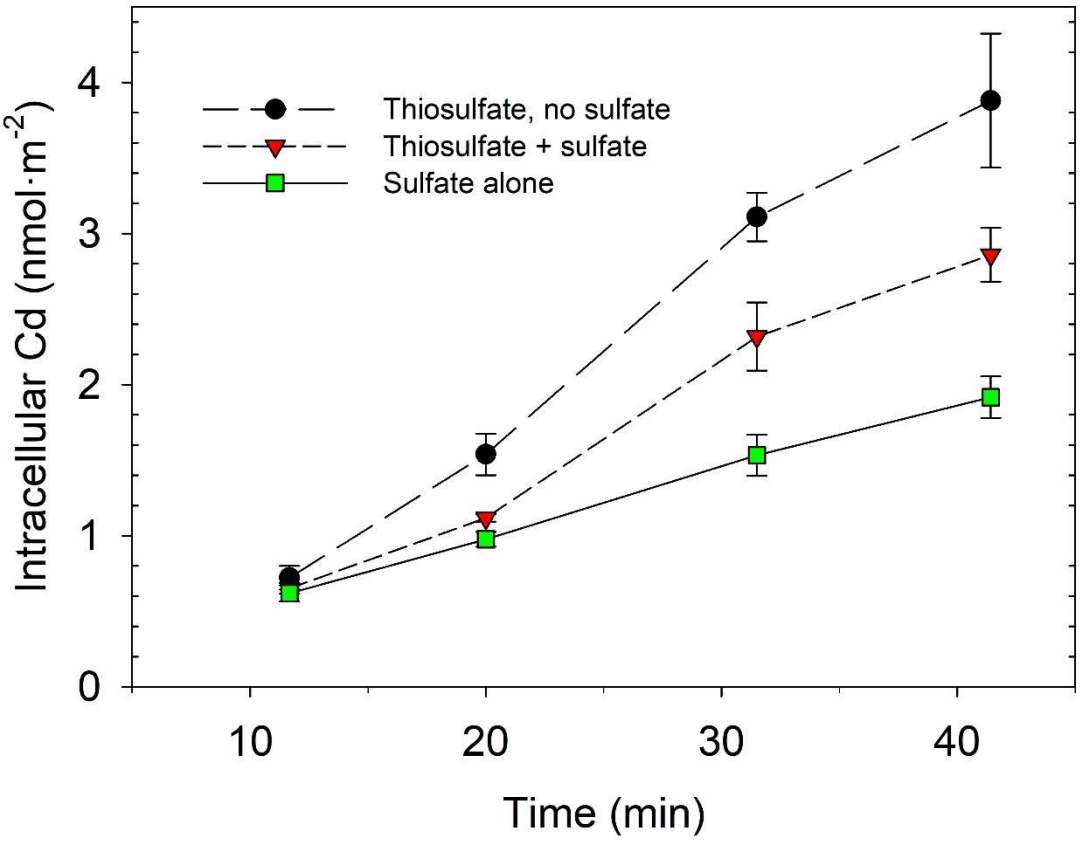

Figure 1: Uptake of Cd by *C. reinhardtii* in the simplified exposure medium containing sulfate alone, sulfate + thiosulfate, or thiosulfate without any sulfate. The error bars represent the standard deviation (n = 3). Details concerning the composition of the three different exposure media can be found in Table 1.

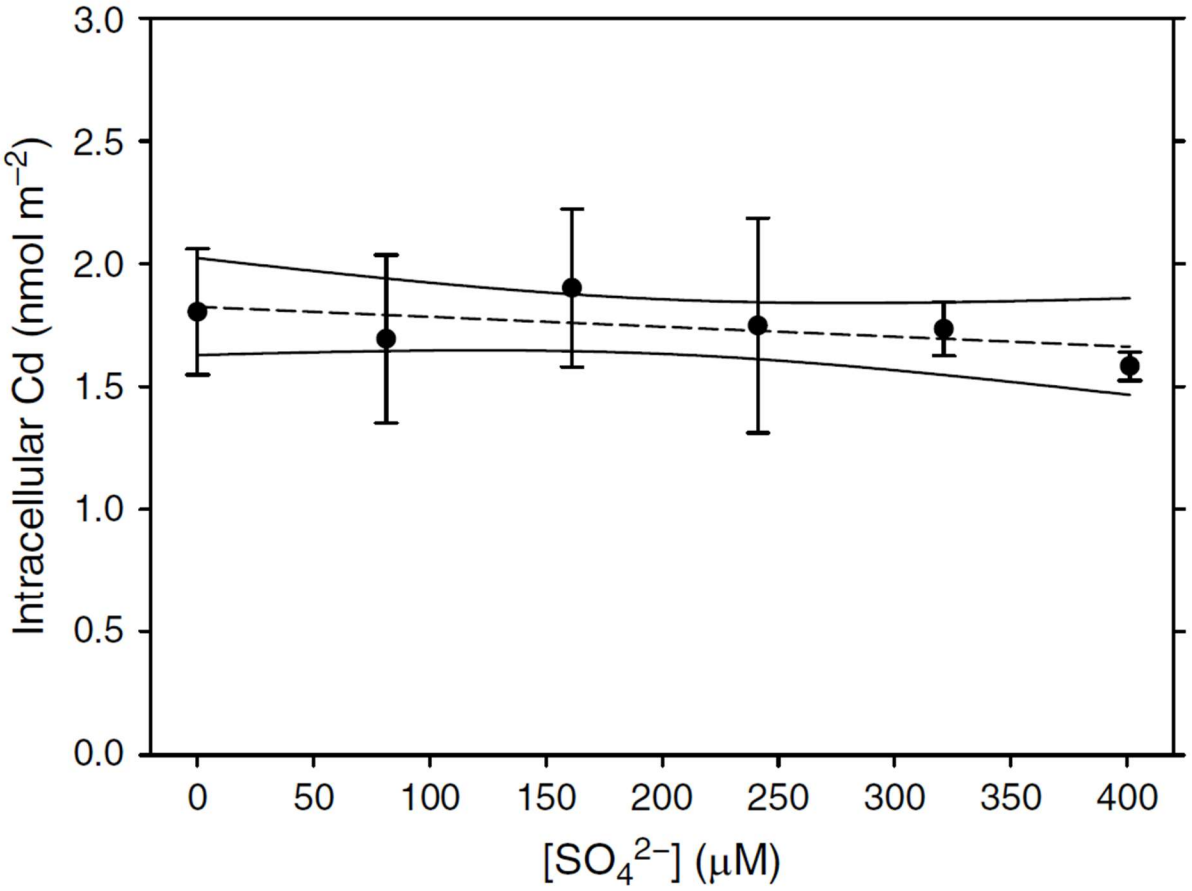

Figure 2: Short-term (30 min) uptake of Cd by *C. reinhardtii* is unaffected by the sulfate concentration if thiosulfate is absent. The error bars represent the standard deviation (n = 3). The concentration of free Cd^{2+} was 3.0 ± 0.1 nM in all media, buffered by NTA in the medium without thiosulfate and by thiosulfate alone in the other medium. Details concerning the composition of the exposure media can be found in Table 1.

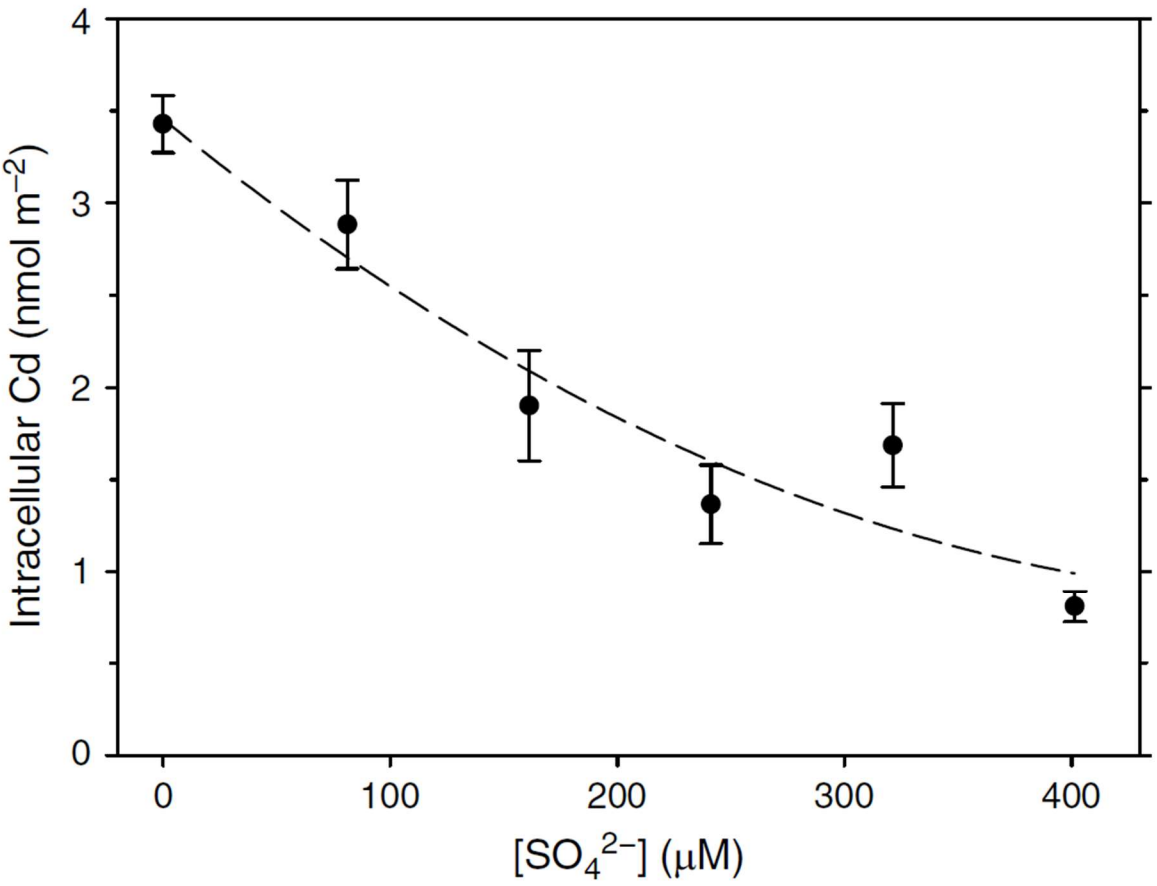

Figure 3: Short-term (30 min) uptake of Cd by *C. reinhardtii* declines as the sulfate concentration is increased in the presence of a fixed thiosulfate concentration (1 mM). The error bars represent the standard deviation (n = 3). Details concerning the composition of the exposure media can be found in Table 1.

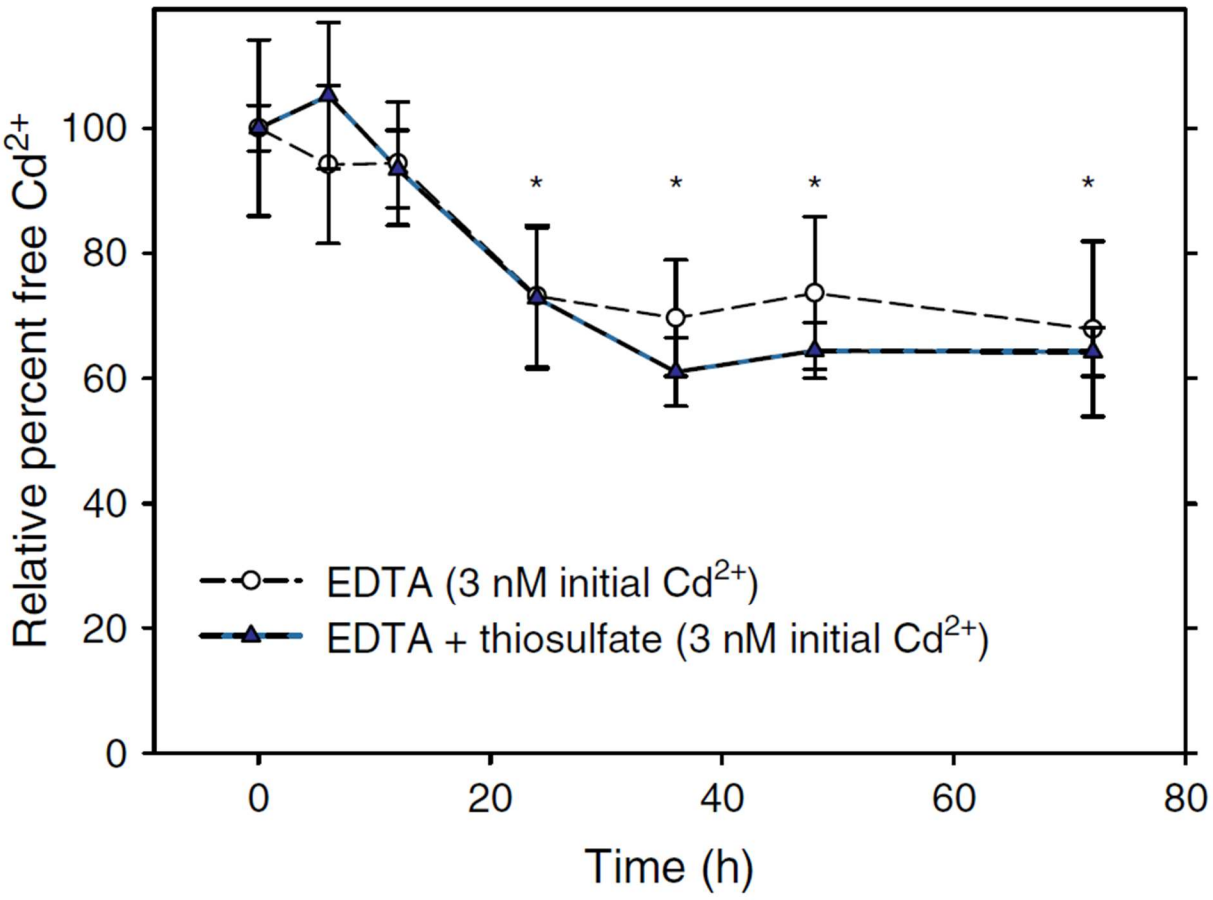

Figure 4: Changes in the relative percentage of free Cd^{2+} in the algal growth medium over the 72-h exposure. The total dissolved Cd concentration decrease by ≤ 10 % over the same time period. The asterisks indicate that the decrease in % free Cd^{2+} was statistically significant ($P < 0.05$). The error bars correspond to the standard deviation (n = 3). Details concerning the composition of the growth medium can be found in Table S1. [EDTA] = 15 µM; [thiosulfate] = 0 or 1 mM.

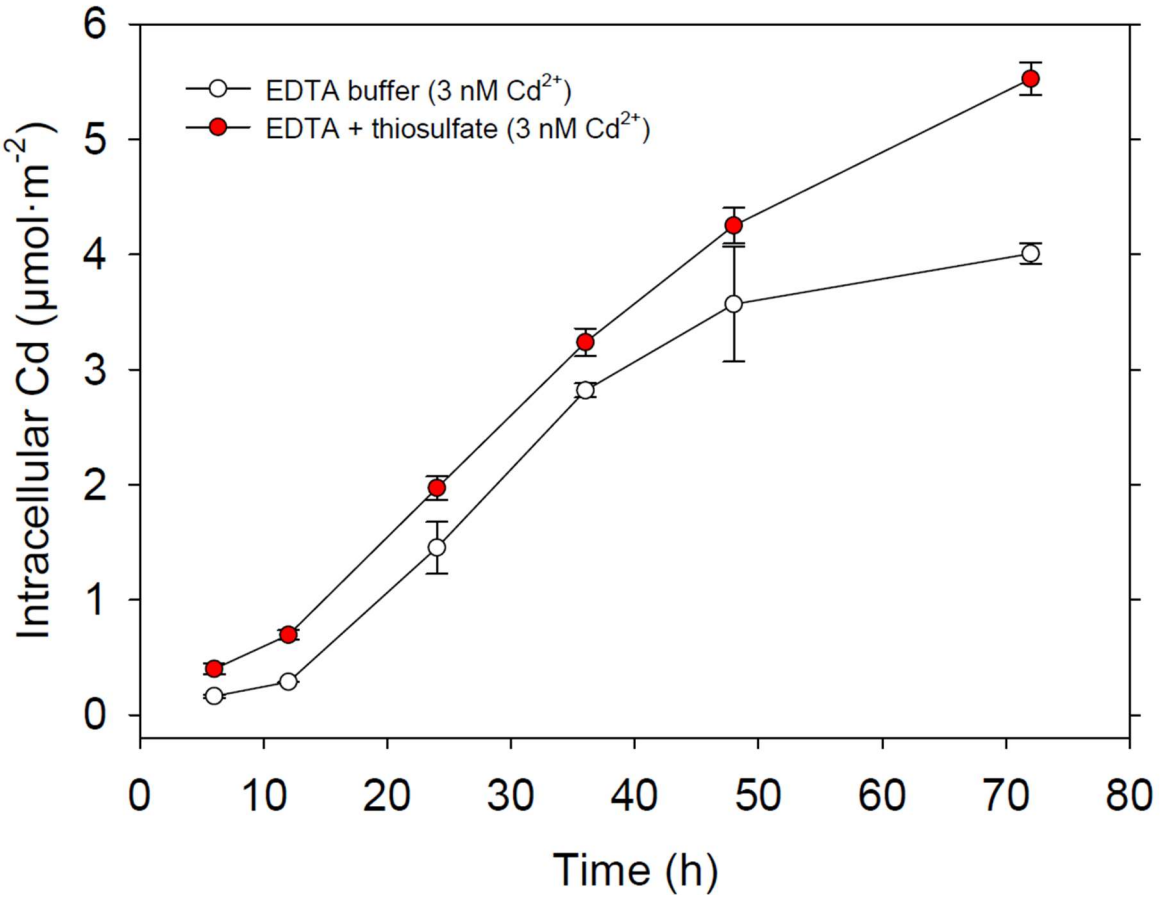

Figure 5: Long-term uptake of Cd by *C. reinhardtii* in exposures with an initial free Cd^{2+} concentration of 3 nM, buffered by EDTA (15 µM) in the absence or presence of thiosulfate (1 mM). The error bars correspond to the standard deviation (n = 3). Details concerning the composition of the growth medium can be found in Table S1.

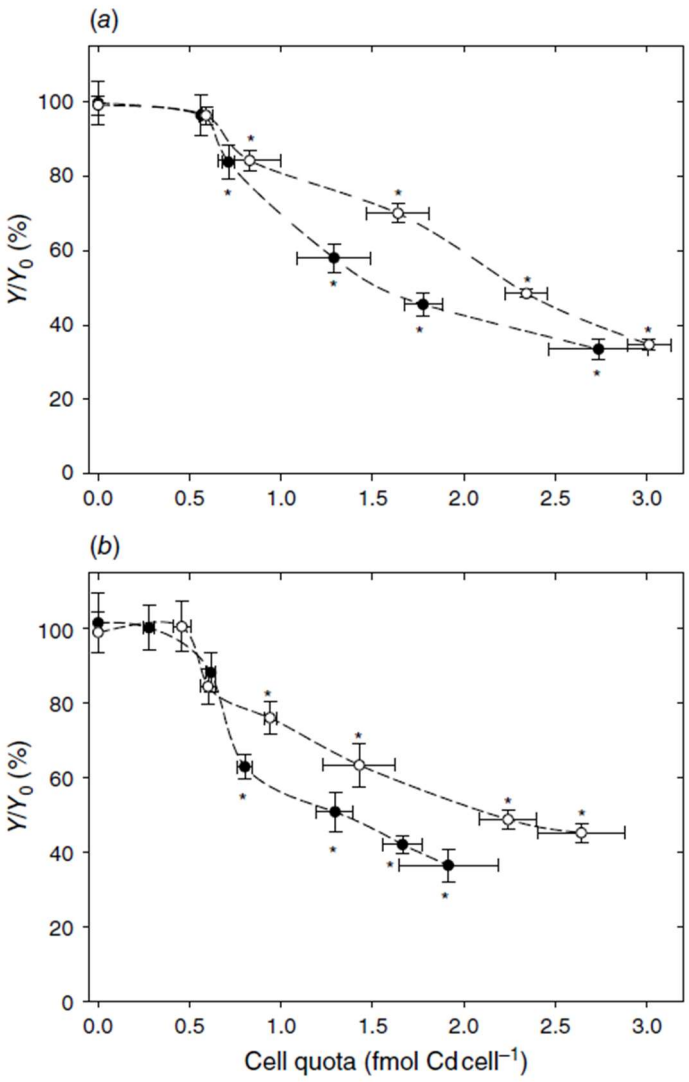

Figure 6: (A) Relative yields of *C. reinhardtii* after 72 h as a function of the calculated cell quota in media buffered with EDTA alone (•) or with EDTA + thiosulfate (o). Series A. EC50 = 2.38 ± 0.16 fmol Cd·cell^{-1} in the medium with thiosulfate + EDTA and EC50 = 1.77 ± 0.24 fmol Cd·cell^{-1} in the medium with EDTA alone. The asterisks indicate exposures that resulted in significantly reduced yields (P < 0.5). The error bars correspond to the standard deviation (n = 3). (B) Relative yields of *C. reinhardtii* after 72 h as a function of the calculated cell quota in media buffered with EDTA alone (•) or with EDTA + thiosulfate (o). Series B. EC50 = 2.16 ± 0.25 fmol Cd·cell^{-1} in the medium with thiosulfate + EDTA and EC50 = 1.35 ± 0.14 fmol Cd·cell^{-1} in the medium with EDTA alone. The asterisks indicate exposures that resulted in significantly reduced yields (P < 0.5). The error bars correspond to the standard deviation (n = 3).

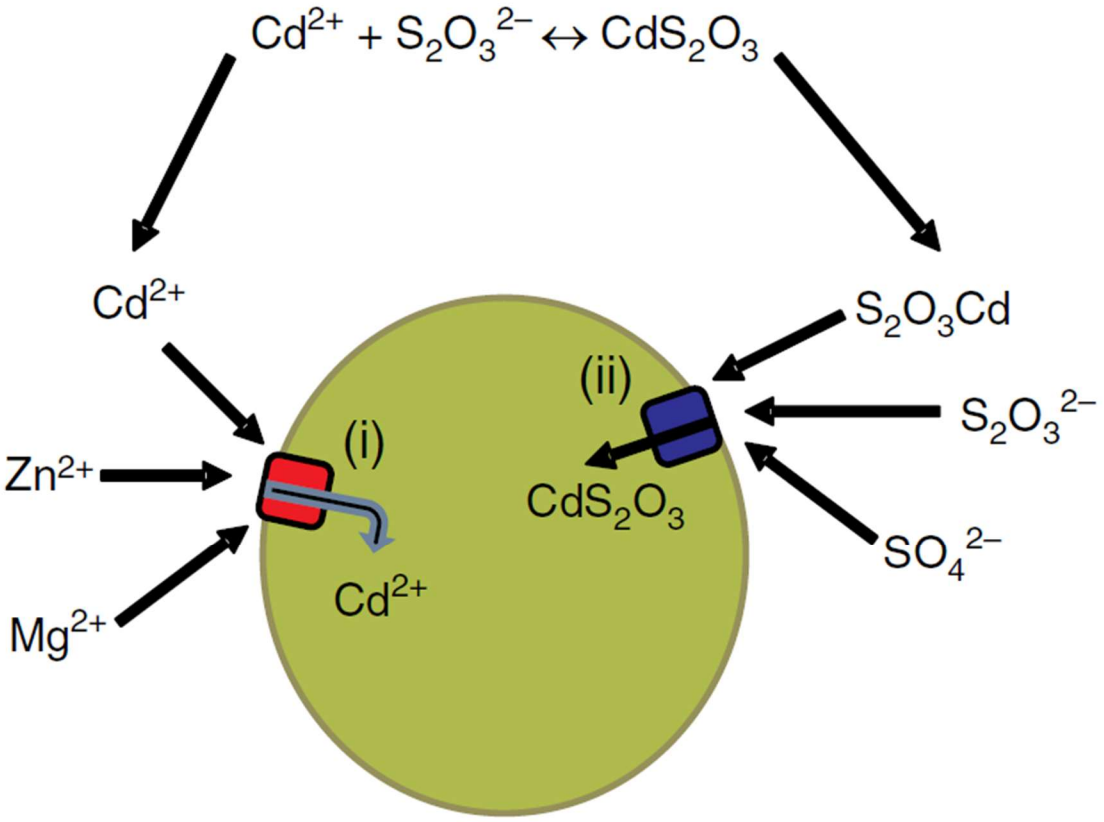

Figure 7: Conceptual model of the possible routes for Cd uptake by *C. reinhardtii* in the presence of thiosulfate. Route (i) depicts the entry of Cd^{2+} via a cation transporter that is normally used by essential cations such as Mn^{2+} and Mg^{2+} (i.e., an example of ionic mimicry). Route (ii) represents the entry of the Cd-S_2O_3 complex via the anion transporter that is known to move sulfate and thiosulfate across the algal cell membrane. The arrows impinging on the transporters represent potential competition for binding to each transporter.