Chemical conditions in the boundary layer surrounding phytoplankton cells modify cadmium bioavailability

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Short title: Metal speciation near cell surfaces

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Abstract

In this study we tested the hypothesis that metal uptake by unicellular algae may be affected by changes in metal speciation in the boundary layer surrounding the algal cells. The freshwater alga *Chlamydomonas reinhardtii* was pre-acclimated to different N nutrition regimes; changes in N nutrition are known to change the nature of extracellular metabolites (e.g., reactive oxygen species 'ROS', and OH^-) and thus boundary layer chemical conditions. Specifically, at a constant bulk free Cd^{2+} concentration, Cd uptake by N-starved algae in cysteine-buffered solution was significantly higher than that in NTA-buffered solution. This enhancement was likely due to an increase of the free Cd^{2+} concentration in the boundary layer, resulting from localized cysteine oxidation by ROS released from these algae. On the other hand, Cd uptake was markedly lower when the free Cd^{2+} concentration near cell surface decreased as a result of an increase in the boundary layer pH of nitrate-acclimated algae or enhanced localized metal complexation. The results imply that redox, acid-base and metal complexation processes in the boundary layer differ from those in bulk water, even under chemically stable bulk conditions, and the boundary layer effect may well be of significance to phytoplankton acquisition of other trace metals.

Keywords: trace metal, phytoplankton, boundary layer, phycosphere, pH, redox status, reactive oxygen species

Introduction

Recently, it has been suggested that low-molecular-weight (LMW) ligands are able to enhance metal uptake by marine phytoplankton, although the means by which phytoplankton acquire metals bound to LMW ligands remain unclear[1-4]. For freshwater phytoplankton, under conditions where the free Cd^{2+}concentration in the bulk solution was held constant, we recently reported that LMW ligands such as cysteine could enhance Cd uptake in the absence of a non-assimilable ligand (i.e., nitrilotriacetic acid, NTA) acting as a metal buffer, but not in its presence[5]. Since the enhanced Cd uptake was not due to uptake of intact Cd-LMW complexes or formation of a ternary surface complex, we concluded that the enhancement was likely associated with an increase in the free Cd^{2+} concentration in the boundary layer[5] – the phycosphere region immediately surrounding an algal cell[6]. However, the specific reactions occurring in this microenvironment surrounding the algal cells remained unclear.

Here, we propose that metal speciation in the boundary layer may differ from that in the bulk solution, due to the redox, pH and metal complexation conditions that prevail in this micro-space near the cell surface. Algae have been shown to release various reactive oxygen species (ROS)[7], and the released ROS have been reported to influence the redox status of bulk Fe and its uptake by algae[8, 9]. However, it remains speculative whether or not boundary layer redox status differs from that in the bulk solution. For large marine diatoms exposed to light, boundary layer pH has been reported to be higher than that of surrounding seawater[10-13], but to our knowledge the influence of this change in local pH on metal bioavailability has not been studied. In addition to this potential pH effect, metal complexation in the phycosphere by locally concentrated algal exudates/metabolites might be more significant than that in bulk water, and such local complexation could reduce the free metal ion concentration, especially when the metal is poorly buffered in the bulk solution. With respect to this latter mechanism, the ecological importance of exudates as a nutrient source for planktonic bacteria living in the phycosphere[6] and the influence of exudates on metal speciation in bulk water[14] have long been recognized. However, it is generally assumed that effect of algal exudates on metal speciation in the boundary layer is the same as that in bulk water.

In this study, short-term uptake of Cd by two strains of a model alga *Chlamydomonas reinhardtii*, pre-acclimated to various conditions of nitrogen nutrition, was studied to verify the putative boundary layer effect; it is well known that changing the forms of nitrogen supplied to an alga leads to changes in the nature of algal metabolites and thus the chemical composition of the boundary layer[15, 16]. Importantly, chemical conditions in the bulk waters, such as the concentrations of free Cd^{2+} and other cations and the pH, were here kept stable by adding metal ligands and a pH buffer and by working at relatively low cell densities to minimize any effect of algal exudates/metabolites on bulk metal speciation. Redox insensitive Cd, an under-appreciated contaminant in freshwater environments[17], was chosen to avoid complications from known surface metal reduction reactions (e.g., Fe and Cu) associated with algal membranes[18, 19].

Materials and Methods

Model algae

Aseptic strains of the freshwater chlorophyte *C. reinhardtii* were used; one strain (CPCC11 wild type mt+, which cannot utilize nitrate for growth) was obtained from the Canadian Phycological Culture Centre (CPCC) of the University of Waterloo) whereas the other strain (CC1690 wild type mt+ [Sager 21 gr], which can utilize nitrate for growth) was obtained from the Chlamydomonas Resource Center of the University of Minnesota. The algae were grown in a modified high salt medium (Table S1) and in a controlled environmental growth chamber (Conviron, CMP3023) with an illumination of 80-100 $\mu mol \cdot m^{-2} \cdot s^{-1}$, at 20 °C and with agitation at ~100-150 rpm.

Modification of N nutrition

In order to change algal metabolites and thus the chemical composition of the boundary layer, different forms of nitrogen source were used to acclimate the algae before the metal uptake tests. The culture media containing different nitrogen sources and the detailed acclimation protocol are described in Note S1.

Briefly, to favor extracellular release of reactive oxygen species (ROS), the algae were acclimated to L-cysteine as the only N source or were N starved for two to four days, given that limited amino acid supply and N starvation have been shown to promote the release of ROS in algae[20]. To favor extracellular release of OH^-, the algae were acclimated to nitrate as the only N source over three months, since nitrate supply is well known to enhance the release of OH^-. Strain CC1690 was employed for nitrate acclimation, and strain CPCC11 was chosen to test localized metal complexation. Following the acclimation period, cells in exponential growth phase were collected for Cd uptake tests on 2-µm polycarbonate filters.

Exposure media

We prepared various exposure solutions for the uptake tests, with addition of different ligands while maintaining the same chemical conditions such as free Cd^{2+}/Ca^{2+}/Mg^{2+}/Na^{+}/K^{+} concentrations, pH and ionic strength; the composition of these exposure media is described in Note S2. Briefly, each exposure medium was a simplified version of the corresponding nitrogen acclimation medium, chosen to avoid physiological changes of the algae during the Cd uptake tests; neither P nor trace metal stock solutions were added to the exposure media, in order to better control Cd speciation. Before use, the exposure media were filtered through 0.2-µm polycarbonate filters (Merck Millipore Ltd.) and carrier free radioactive ^{109}Cd (365 or 387 $Ci \cdot g^{-1}$, two batches, Eckert & Ziegler, California) was used to follow Cd uptake at 0.1 or 1 nM free Cd^{2+}. These concentrations, comparable to those determined in lake waters on the Canadian Precambrian Shield[21], were set by adjustment of the appropriate ligand concentration in solutions with a total Cd concentration of 20 nM. The added ^{109}Cd was less than 1% of total Cd in the exposure solutions, and the activities ranged from 1.33 to 1.62 $kBq \cdot mL^{-1}$ medium (i.e., 0.13 to 0.15 nM ^{109}Cd). In the solution without any ligand (i.e., 1.42 nM total Cd, 1 nM free Cd^{2+}), the radioactivity was 0.17 $kBq \cdot mL^{-1}$ medium (i.e., 15.9 pM ^{109}Cd).

Cysteine and NTA were used as the model ligands, for several reasons: neither Cd-cysteine nor Cd-NTA complexes are assimilable by our algae under our experimental conditions[5]; cysteine is more sensitive to oxidation than NTA, which facilitated the investigation of boundary layer oxidation; the different protonation constants of the two ligands result in different changes in the free Cd^{2+} concentration for a given change in the boundary layer pH.

Efforts were made to minimize the danger of cysteine oxidation or any other unknown chemical changes in the exposure media: (i) cysteine/NTA stock solutions were prepared daily, and the cysteine solution was flushed with N_2 gas; (ii) cysteine concentrations in the exposure media (typically 75 μM), as determined by the Ellman test, were identical to the nominal values; and (iii) in the filtrates obtained after 1-h Cd uptake tests, no oxidation of cysteine and no change in Cd speciation was observed, compared to the original exposure solutions[5].

Exposure media were pre-equilibrated for at least 14 h. Unless otherwise indicated, the pH of the exposure media was buffered with 10 mM 3-(N-morpholino)-propanesulfonic acid (MOPS) at 7.0, and verified just before carrying out uptake tests. We collected 1-2 mL subsamples of the exposure media to determine the ^{109}Cd radioactivity.

Calculation and determination of Cd speciation

The speciation of Cd in the exposure media was calculated with chemical equilibrium software (MINEQL+ v4.62)[22] with updated stability constants, and the calculation details are described in Note S1. To verify the calculation of Cd speciation, we also quantified the free Cd^{2+} concentrations in selected exposure media by using an ion-exchange technique (IET)[23]; the protocol is described in Note S3. In the solutions containing cysteine, we did not quantify the free Cd^{2+} concentration; preliminary tests indicated that $CdHCys^{+}$ binds electrostatically to the sulfonic acid resin, leading to an over-estimation of the free Cd^{2+} concentration.

Short-term Cd uptake

For a given uptake test, all the algal cells came from the same algal batch culture, grown under a specific N nutrition regime. For example, to test boundary layer redox conditions, the cysteine-acclimated (or N-starved) algae were harvested, rinsed and resuspended into exposure solutions buffered by either cysteine or NTA, and during a one-hour exposure period algal samples were collected two to five times. The Cd associated with the cells after washing by a solution containing 1 mM EDTA was considered as the internalized metal, and details of the protocol are described in Note S1. Cadmium uptake rates were linear over the first one hour under the tested conditions (Fig. S1).

In some experiments, we collected water samples, just after collection of the algae by filtration and before washing them with the EDTA solution. These filtrates were further filtered through 0.2-µm polycarbonate filters in order to confirm the constancy of the concentrations of total Cd and free Cd^{2+} (determined by the IET) during the uptake period.

Similarly, to examine whether or not extracellular ROS (e.g., O_2^- and H_2O_2) were involved in the boundary layer oxidation of cysteine, the uptake test was repeated in the presence of ROS scavengers (superoxide dismutase and catalase) or an ROS stimulator (β-nicotinamide adenine dinucleotide - reduced form (NADH)). Nitrate-acclimated algae (strain CC1690) were exposed to solutions buffered either by NTA or cysteine to test the boundary layer pH effect, whereas ammonium-acclimated algae (strain CPCC11) were exposed to solutions with or without NTA to test for localized metal complexation effects.

Measurement of ^{109}Cd and calculation of Cd uptake rate

The radioactivity of the collected samples was determined with a gamma counter (Wallac Wizard2, Perkin Elmer). The samples were placed in glass counting vials filled with 5 mL water to minimize sample geometry effects and each sample was counted for 10 min under the following settings: counting window (Dynamic) peak position, 22 keV; low boundary, 16 keV; high boundary, 32 keV; counting spectrum type, single peak.

Cadmium uptake was calculated from the linear regression of Cd accumulated by the cells over time and has been normalized on the basis of the cell surface area. Uptake was based upon the measured radioactivity in the algae and the specific radioactivity of Cd in the exposure solution. The activity of the lower filter was subtracted from that measured on the upper filter to correct for passive retention of radiolabeled Cd by the filters.

Estimation of boundary layer pH

Direct measurement of the pH in algal boundary layers is technically difficult; the spatial resolution attainable with the finest available pH microelectrode (10 µm tip) is around 100 µm (Unisense, Denmark), i.e., about ten times higher than the thickness of the algal boundary layer. However, theoretically, if extracellular production of OH^- by algae can modify the pH in the boundary layer, there would also be a measurable pH change in the bulk solution over time. Increasing the algal cell concentration, elimination of the pH buffer and increasing the exposure time would facilitate the measurement of changes in extracellular pH in the bulk solution. Accordingly, tests were performed in the presence of high concentrations of algal cells with/without addition of pH buffer over both short-term (hours) and long-term (days) periods; identical solutions without algae were used as the controls. In the short-term test with addition of pH buffer, the concentration of bicarbonate was increased to 5 mM to avoid its photosynthetic depletion at such high cell densities.

These tests were carried out with the same exposure solutions as for the uptake tests (but without addition of Cd and ligands) under identical experimental conditions (i.e., light, agitation, temperature and open to air), unless otherwise indicated. A freshly calibrated pH electrode (Accumet™, Fisher Scientific) was placed directly in the test samples with/without pre-rinsed algae.

Treatment effect and experimental reproducibility

All experiments designed to identify treatment effects (i.e., addition of NTA or cysteine, or no ligand) were run with the same algal batch, pre-acclimated to the same N source (i.e., the same population of algal cells was used for the different treatments), and thus for a given test any difference in metal uptake rates is not associated with biological factors such as a batch-to-batch variation in the membrane-bound transport system for the metal. Moreover, the majority of the tests (typically each with three replicates) were repeated with different algal batches two to five times to ascertain the reproducibility of the results (Table S2).

Statistical analyses

The SPSS 16.0 and SigmaPlot 12.5 software packages were used to analyze data. The Cd uptake rates were compared by using a general linear model in SPSS, whereas linear regressions for the Cd uptake rates over one hour were calculated with SigmaPlot. The significance level was set at $p < 0.05$, unless otherwise indicated.

Results

Cd uptake in the presence of cysteine or NTA by N-starved and cysteine-acclimated algae and influence of addition of ROS scavengers or stimulator

At the same bulk [Cd^{2+}], the Cd uptake rate by N-starved algae in the presence of cysteine was 4.0-times higher than that in the presence of NTA (Fig. 1A). As predicted from the proposed boundary cysteine oxidation effect, the enhancement disappeared when the N-starved algae were treated with the ROS scavengers superoxide dismutase and catalase (Fig. 1B), the addition of which was designed to suppress cysteine oxidation by ROS. On the other hand, the enhancement in Cd uptake was unaffected when the N-starved algae were treated with NADH (Fig.S2), although this treatment has been shown to stimulate O_2^- production in marine bacteria[24].

Consistent with our previous study[5] and the boundary oxidation effect, the Cd uptake rate by cysteine-acclimated algae in the presence of cysteine was also higher than that in the presence of NTA, at the same bulk [Cd^{2+}] (Table S2). As predicted by free ion activity model[25], the Cd uptake rate by the cysteine-acclimated cells decreased when the concentration of free Cd^{2+} in the bulk solution was lowered by adding either more NTA or cysteine into the solutions containing 20 nM total Cd (Fig. 2). However, we also observed that for the comparable uptake rates, the bulk concentrations of free Cd^{2+} in the cysteine-buffered solutions were only half of those in the NTA buffered solutions. Based upon these measurements, we were able to estimate the concentration of newly liberated Cd^{2+} and cysteine oxidation rate in the boundary layer (see the Discussion below).

Cd uptake in the presence of cysteine or NTA by NO_3^--acclimated algae and simulated effect of boundary layer pH enhancement on Cd speciation

Assuming that the pH in the boundary layer of the NO_3^-- acclimated cells would be consistently higher (i.e., > 7.0) than in the bulk solution (pH = 7.0), we ran chemical equilibrium simulations with MINEQL software to calculate how an increase in pH would affect Cd speciation in the boundary layer. The calculations indicated that the effect of pH increases on $[Cd^{2+}]$ would be greater in the cysteine-buffered system than in the NTA-buffered solutions, i.e., $[Cd^{2+}]$ would decrease more and the algae would take up less Cd under the cysteine-buffered conditions (Fig. 3A), which would be markedly different from those observations on the N-starved or cysteine-acclimated algae.

Consistent with this prediction, at a constant $[Cd^{2+}]$ of 0.1 nM in the well-buffered bulk solution, the Cd uptake rate in the presence of cysteine by NO_3^--acclimated algae was reduced to 25% of that observed in the presence of NTA (Fig. 3B). Note that the pH of the exposure solutions was verified before and after the test and it remained constant at pH 7.0, buffered by MOPS. The lower Cd uptake rate in the cysteine-buffered solution also indicates any oxidation of cysteine in the bulk medium was negligible; otherwise, higher Cd uptake rates in the presence of cysteine would have been observed.

Consistent with the literature[16], for the NO_3^--acclimated algae at very high cell densities, the extracellular pH rose despite the presence of the pH buffer both in the short-term and in the long-term exposure (Fig. S3). In the absence of the pH buffer, the extracellular pH rose much higher and more rapidly within a few hours (Fig. 3C).

Comparison of Cd uptake from solutions with or without a metal-complexing ligand and the IET-measured free Cd^{2+} concentrations in bulk solutions

To test for boundary metal complexation, one has to maintain the free Cd^{2+} concentration in the ligand-free solution constant during the uptake period; accordingly, relatively low cell densities and a relatively high bulk free Cd^{2+} concentration (1 nM) were used. Specifically, at 1 nM bulk Cd^{2+}, the Cd uptake rate by the ammonium-acclimated algae (strain CPCC11) in solutions without addition of any metal-binding ligand was only 19% of the uptake rate in solutions containing NTA (Fig. 4A). Similarly, a significantly lower uptake rate in the absence of a ligand was observed in comparison to the uptake rates observed in the presence of either L- or D-cysteine (Fig. S4); the unnatural D-isomer was used to eliminate the possible facilitated uptake of cysteine-Cd complexes or any other direct biological effect.

Importantly, in the filtrates collected during the uptake tests, the determined concentration of Cd^{2+} in the absence of a ligand (0.83 ± 0.14 nM) was not lower than that in the presence of NTA (0.58 ± 0.06 nM) (Fig. 4B). The free Cd^{2+} concentrations in solution before adding algae, as determined by the IET, were very close to the values calculated with MINEQL. Specifically, in the solutions containing NTA, at the calculated 1.13 nM Cd^{2+}, the determined value was 0.91 ± 0.09 nM (N = 3, mean ± SD) (Table S3).

Discussion

The present work demonstrates unexpected variability (i.e., consistent increase or decrease) in Cd uptake at the same bulk Cd^{2+} concentration by a given batch of the model freshwater alga *C.*

reinhardtii in the presence of different ligands (i.e., cysteine vs. NTA). Interestingly, the variability in Cd uptake was closely associated with the forms of nitrogen supplied for the algae rather than the Cd chemistry in the bulk solutions. Moreover, in the absence of a metal-binding ligand at a fixed bulk Cd^{2+} concentration and for a given algal culture, we observed Cd uptake rates that were unexpectedly lower than those in the presence of NTA. All of the results can be explained by changes in the boundary layer chemical conditions (redox, pH and metal complexation), but not by other hypotheses, as discussed below.

Is the enhanced Cd uptake by N-starved or cysteine-acclimated algae, in the presence of cysteine, due to cysteine oxidation by released ROS in the boundary layer?

As demonstrated in our earlier work[5], the enhanced Cd uptake rates by cysteine pre-acclimated or N-starved algae in the cysteine-buffered solution, in comparison to that in the NTA-buffered solution, cannot be explained by cysteine oxidation in bulk solution, by diffusion limitation of the supply of free Cd^{2+} from the bulk solution to the algal surface, by the uptake of intact Cd-cysteine complexes or by the formation of ternary surface complexes. The enhancement effect in the presence of cysteine was linked to the N-starvation and cysteine pre-acclimation. Nitrogen substrate[18, 26] and cell physiological state[27] have been shown to affect metal uptake rates, but these effects on metal uptake are mainly due to N-associated or metal-induced biological differences such as activity of membrane-bound redox enzymes or the expression of transmembrane metal transporters, as observed for different N- or metal-acclimated algal batches. In our study, however, we compared short-term Cd uptake rates in exactly the same algal cells, exposed to different ligands; changes in membrane-bound redox enzymes or metal transporters would not be expected to occur during the one-hour uptake tests.

We suggest that the enhanced Cd uptake in the presence of cysteine is associated with a higher [Cd^{2+}] in the boundary layer of cysteine-acclimated or N-starved algae, and that the higher [Cd^{2+}] results from localized oxidation of Cd-cysteine complexes involving algal metabolites (e.g., ROS) and subsequent liberation of Cd^{2+}. In contrast, the boundary [Cd^{2+}] in the presence of NTA would change little given that NTA is less sensitive than cysteine to ROS oxidation. If ROS were involved in changing Cd speciation in the boundary layer, the presence of extracellular ROS scavengers would inhibit the cysteine oxidation and liberation of Cd^{2+} from the ligand. Indeed, with the N-starved algae, we found that the enhancement effect on Cd uptake disappeared in the presence of two well-known ROS scavengers (i.e., SOD and catalase). In our study, superoxide (O_2^-) likely plays a minor role in the transformation of Cd-cysteine complexes since the enhanced Cd uptake was little affected by the addition of NADH, which is known to stimulate the production of O_2^- [24, 28].

Although we did not directly quantify extracellular production of ROS, algae continuously release them into external space[7] and the extracellular production of H_2O_2 can be enhanced by nitrogen starvation or utilization of amino acids but inhibited by the supply of ammonium nitrogen[20]. Thus, more ROS would be produced by N-starved (or cysteine-acclimated) algae than by NH_4^+-acclimated cells, which would result in more oxidation of cysteine (and thus a higher [Cd^{2+}]) in the boundary layer. This idea is in agreement with the enhanced Cd uptake by the N-starved (or cysteine-acclimated) algae observed in this study and the absence of an enhancement in NH_4^+-acclimated cells, which was reported in our previous study[5] (Table S2).

The boundary layer oxidation effect might also exist for other microorganisms in both fresh and ocean waters, since the enhancement of metal uptake in the presence of cysteine was also observed in another freshwater green alga (*Pseudokirchneriella subcapitata*)[5], a freshwater cyanobacterium (*Anabaena flos-aquae*)[5], marine diatoms[1, 3, 29] and indigenous marine phytoplankton[30]. It has been shown that the extracellular production rate of ROS varies greatly among algae species and is influenced by light conditions and ambient trace metals[7, 31].

One important question is whether or not boundary layer oxidation could result in a significant change in the free metal ion concentration; how large would the difference in free Cd^{2+} concentrations be between bulk water and cell surface? To estimate this difference, we assumed that there was no change in boundary [Cd^{2+}] in the presence of NTA, and the increment in Cd uptake in the presence of cysteine was completely due to an increase in boundary [Cd^{2+}] by boundary cysteine oxidation. Our calculation indicates that the concentration of free Cd^{2+} in the boundary layer of the cysteine-acclimated algae in the cysteine-buffered solution was twice as high as that in the bulk solution (Note S4).

Based upon the same assumptions, our calculated oxidation rate of cysteine in the boundary layer is 2×10^{-16} mol·cell^{-1}·h^{-1} (Note S5). Given that 2 moles of cysteine are consumed per mole of H_2O_2 [32], the estimated H_2O_2 consumption rate in the boundary layer of cysteine-acclimated *C. reinhardtii* would be 1×10^{-16} mol·cell^{-1}·h^{-1} (i.e., 1×10^{6} molecules H_2O_2·cell^{-1}·min^{-1}), which is similar to the maximal production rate (i.e., 7×10^{5} molecules H_2O_2·cell^{-1}·min^{-1}) in bulk solution by *C. reinhardtii* as reported by Suárez et al. for different experimental scenarios[31]. It also compares well with the recently reported extracellular H_2O_2 production rates (0.6-14 × 10^{-16}

mol·cell^{-1}·h^{-1}) for marine phytoplankton[7]. The calculation also shows that cysteine oxidation would have little influence on Cd speciation in the bulk solution but could significantly affect Cd speciation in the boundary layer (Note S5). Thus, oxidation of cysteine in the bulk solution would be undetectable during the short-term uptake period; this is consistent with the results of our direct measurement of cysteine concentrations in the exposure solutions.

In addition to N supply, we speculate that nutritional supplies of minor nutrients such as Zn or Cu might also facilitate boundary layer oxidation effects by changing extracellular release of ROS and/or surface SOD synthesis. In experiments on marine phytoplankton[1, 3], enhancement in metal uptake in the presence of cysteine was more dramatic in Zn/Cu limited cells than in metal-replete cells. In our study, all algal cultures grew under metal-replete conditions, and trace metals other than Cd were not added to the exposure media, which rules out any effect of other metals on Cd uptake (e.g., Zn contamination in ligand solutions; see details in Supporting Information, section on '*Calculation of Cd speciation*').

Is the reduced Cd uptake due to alkalization of the boundary layer in NO_3^--acclimated algae?

It is well known that algal utilization of NO_3^- results in release of hydroxide HO^- ions[16] and we also observed that the pH rose in the exposure solutions after adding a very high concentration of NO_3^--assimilating algae. It has been reported that the cell surface pH in marine algae can be higher than the well-buffered bulk seawater pH, by 0.4 to 1.0 units[10, 11], the local pH enhancement being attributed to both nitrate utilization (i.e., the coupling to extracellular release of OH^-)[16] and photosynthesis (i.e., removal of intracellular carbon dioxide by the carboxylase reaction of Rubisco decreases external carbon dioxide and consequently increases the external pH)[33].

Assuming the boundary layer pH of our nitrate-fed algae was higher than 7.0, chemical equilibrium calculations show that the [Cd^{2+}] would decrease more under the cysteine-buffered condition than the NTA-buffered condition, due to differential protonation of the ligands. Given the absence of changes in the bulk solution pH, it is then reasonable that the lower [Cd^{2+}] in the boundary layer in the cysteine-buffered system would result in a lower Cd uptake rate than in the NTA-buffered system; localized *alkalization* can thus explain the 'lower than expected' uptake of Cd by the NO_3^--acclimated algae.

Is the reduced Cd uptake observed in the absence of a ligand due to metal complexation in the boundary layer?

In addition to possible redox and pH changes near the cell surface, as discussed to this point, we also suspect that metal sequestration in this microenvironment might differ from that in the bulk waters, since algal exudates are known to be enriched in the phycosphere. In what appears to be an example of this type of effect, Cd uptake from an exposure solution with no added ligand was markedly lower than uptake in the presence of NTA, although the bulk Cd^{2+} concentrations were the same in both exposure media (Figure 4). We attribute the lower Cd uptake rate observed in the absence of a metal buffer to the presence of a lower [Cd^{2+}] in the boundary layer. Specifically, the lower [Cd^{2+}] would be due to Cd sequestration by exuded algal ligands including cell wall proteins[34], polysaccharides and other unknown metal-binding ligands present in this microenvironment, rather than to the potential boundary layer redox/pH effects. In the presence of NTA, Cd^{2+} was well buffered and the possible local complexation by algal exudates would influence the free Cd^{2+} concentration to a much lesser extent.

Note that the reduced Cd uptake in the absence of NTA cannot be due to a decrease in the free Cd^{2+} concentration in the bulk solution. In the absence of NTA, we added slightly more Cd than the theoretically required amount, to make up for the small decrease in dissolved bulk Cd concentration that occurs during the one-hour uptake test (due to metal adsorption and uptake by the algal cells). The measurement of free Cd^{2+} in the uptake filtrates with the IET confirmed that the free Cd^{2+} concentration in the absence of NTA was comparable to (actually slightly higher than) that in the presence of NTA (Table S3).

From a geochemical perspective, it is reasonable to assume that metal-complexing ligands exuded into the boundary layer would sequester free metal ions including Cd^{2+} and thus reduce $[Cd^{2+}]$ to a lower concentration than in the ambient water; the extent of this reduction would depend upon the concentration of the unknown ligands and their affinity for metal ions. Theoretically, the concentration of algal exudates in the phycosphere would never be equilibrated with bulk water; the highest concentration of the exudates (i.e., hot spots) would be in the boundary layer since they are excreted during normal metabolism of the cells[35]. A concentration gradient of exudates is suspected to exist surrounding the cell, and consequently the $[Cd^{2+}]$ would decrease close to the cell membrane.

In the present case, the relatively higher Cd uptake in the presence of NTA (Figure 4A) cannot be associated with a possible boundary pH effect. Specifically, we observed that there was a small pH increase in a solution without the pH buffer in the presence of a high concentration of these NH_4^+-acclimated algae; an increase in the boundary pH would lower boundary Cd^{2+} and thus its uptake in the presence of NTA (Fig. S5), ruling out the importance of boundary layer pH changes in this case. Although NH_4^+ utilization can result in hydrogen ion efflux, algal photosynthesis (i.e., draw-down of external carbon dioxide) can overcome this external pH effect related to NH_4^+ uptake and assimilation (*J. Raven, pers. comm.*). Thus, we conclude that in poorly buffered fresh waters (e.g., waters with few if any metal-binding ligands)[21], the dominant species of dissolved Cd would be Cd^{2+} and localized complexation by algae would result in lower uptake of Cd than that predicted from the bulk [Cd^{2+}].

Interaction of three factors in the boundary layer

In addition to the ambient water chemistry, the free Cd^{2+} concentration in the phycosphere will be determined by the overall effect of algal metabolites/exudates (oxidants/reductants, bases/acids, and metal-binding ligands) on Cd speciation. In the bulk water the three factors are similarly interrelated and interact with each other, but in the boundary layer they are mainly driven by algal metabolism. When the respective effects of the three factors on Cd speciation cancel out effects (i.e., the overall effect is negligible), the Cd uptake rate would change little. Because the boundary layer effect stems from algal metabolism, the relative importance of one factor versus another would depend upon the dominant metabolite/exudate species. For instance, when there is significant extracellular ROS exudation and little H^+/OH^- release, the redox change would dominate the overall boundary effect on Cd speciation, and vice versa.

The relative importance of one factor versus another in affecting boundary layer Cd speciation would also depend upon the nature of the metal-binding ligands occurring in the bulk solution. For example, when the metal is dominantly sequestered by redox-insensitive ligands (e.g., NTA and EDTA), Cd speciation would be little affected by any redox changes in the boundary layer whereas when the metal is bound to ligands whose dissociation from Cd is independent of pH, the boundary layer pH change would play a negligible role in affecting the Cd speciation near the cell surface. However, interactions between trace metals and natural ligands are often redox-sensitive and pH-dependent, and some trace metals (e.g., Fe, Cu, Mn, etc.) are themselves sensitive to redox changes.

Implications

Unlike its biological role in marine diatoms[36], the uptake of Cd in our freshwater algae was likely accidental; the enhanced or decreased uptake of Cd in our algae was not due to up- or down-regulation of metal transport in response to micronutrient requirements but likely resulted from changes in its speciation near cell surface, which was influenced by algal metabolites/exudates. The present study highlights the importance of free-metal ion activities in the boundary layer in determining the uptake (nutrition and toxicity) of cationic trace metals and the importance of biological processes or physiology in affecting metal speciation near the cell surface. Knowledge about metal speciation in bulk waters and membrane-associated surface reactions is not enough to depict the whole picture of metal acquisition, and direct quantification of boundary layer chemical conditions would help bridge this knowledge gap.

Phytoplankton actively exude chemical/biological substances into their extracellular microenvironments during normal growth, and distinct gradients in pH, dissolved oxygen and other chemical conditions have been characterized at the micrometer scale in the boundary layer of some phytoplankton species (e.g., *Trichodesmium* and diatoms) [13, 37]. The resultant boundary layer effect is likely of significance to phytoplankton not only for uptake of Cd but also for their acquisition of essential trace metals such as Fe, Cu and Zn, given that their speciation is also pH and/or redox sensitive.

The present work was carried out on model algae in the absence of bacteria. However, in nature, bacteria frequently cluster near, or attach to, phytoplankton, but the potential influence of bacteria-phytoplankton interactions in the boundary layer on metal bioavailability is little examined[38]. Does bacterial metabolism itself alter chemical conditions in the phycosphere (e.g., CO_2 release from respiration and redox reactions)? Do bacteria degrade or exude metal-binding ligands in the micro-space? If true, changes in metal speciation in this microenvironment would not be caught by bulk water analyses, but knowledge of such changes would be indispensable for understanding trace metal bioavailability.

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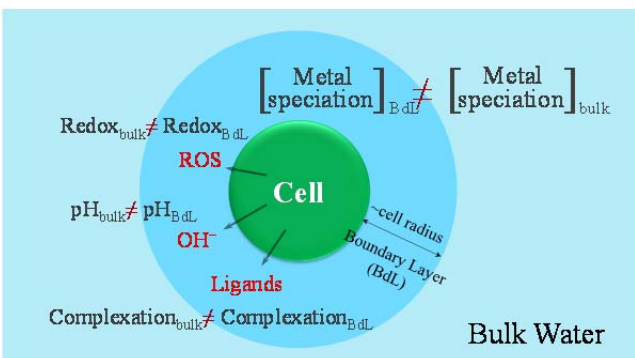
Supporting Information

Preparation and chemical composition of culture and exposure media, and rinse solutions. Modification of algal N nutrition. Estimation of cysteine oxidation rate and the concentration of newly liberated Cd^{2+} in the boundary layer. Tabular summary of one-hour Cd uptake rates by two strains of *C. reinhardtii*. Determination of free Cd^{2+}concentrations by the ion-exchange technique. Figures showing linearity of one-hour uptake of Cd by algal cells acclimated under different conditions and Cd uptake in the presence of NADH and D-cysteine. Figures showing the time-course of extracellular release of OH^- from algae.

Figures



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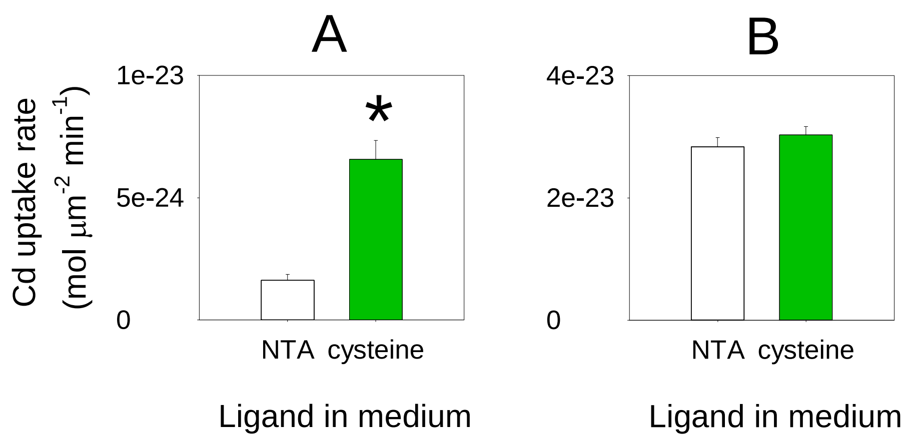

Figure 1. (**A**) Higher Cd uptake rate in the presence of cysteine than that in the presence of NTA at 0.1 nM bulk Cd^{2+} by N-starved algae (CPCC11) at 1.0×10^5 cells·mL^{-1} ($p < 0.05$); and (**B**) Similar Cd uptake rates at 0.1 nM bulk Cd^{2+} (buffered by NTA or cysteine) in the presence of superoxide dismutase and catalase by N-starved algae (CPCC11) at 2.1×10^5 cells·mL^{-1} ($p > 0.05$). N = 2-3, mean ± SEM. The asterisk above the bar indicates a significant difference.

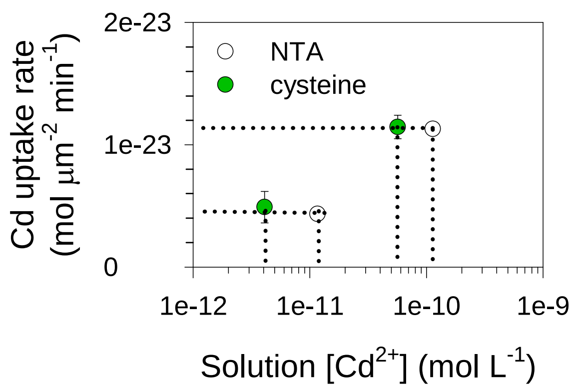

Figure 2. Cd uptake rates at different bulk [Cd^{2+}] in the presence of NTA or cysteine by cysteine-acclimated algae (CPCC11) at 1.0×10^5 cells·mL^{-1}. N = 2-3, mean ± SEM. The dotted lines are drawn to show the differences in bulk [Cd^{2+}] at comparable Cd uptake rates. Exposure medium: total Cd = 20 nM, NTA = 10 or 100 µM, and cysteine = 113 or 450 µM.

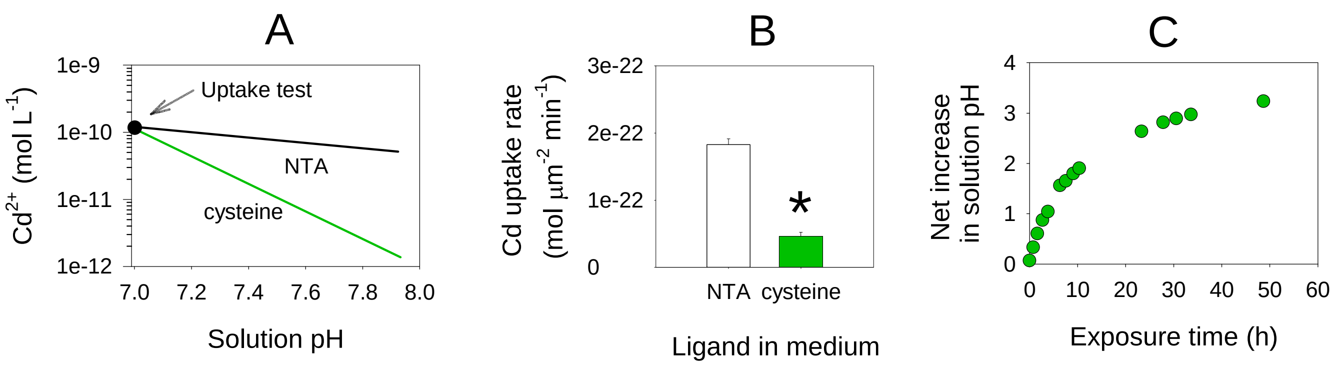

Figure 3. (A) Simulation of the change in free Cd^{2+} concentration with increasing pH in NTA- or cysteine-buffered media (total Cd = 20 nM, the initial [Cd^{2+}] = 0.1 nM at pH 7.0). Note that the effect of the pH change is greater for the cysteine-buffered system than for the NTA-buffered system. (B) Cd uptake rate (n = 3, mean ± SEM) at 0.1 nM bulk Cd^{2+} in the presence of NTA or cysteine (solution pH was buffered at 7.0 with 10 mM MOPS) by NO_3^--acclimated *C. reinhardtii* (CC1690) at 6.0-6.8 × 10^4 cells·mL^{-1}; (C) pH change in bulk solution (no addition of pH buffer, in order to detect short-term pH changes) containing NO_3^--acclimated algae (CC1690) at 2.8 × 10^5 cells·mL^{-1}.

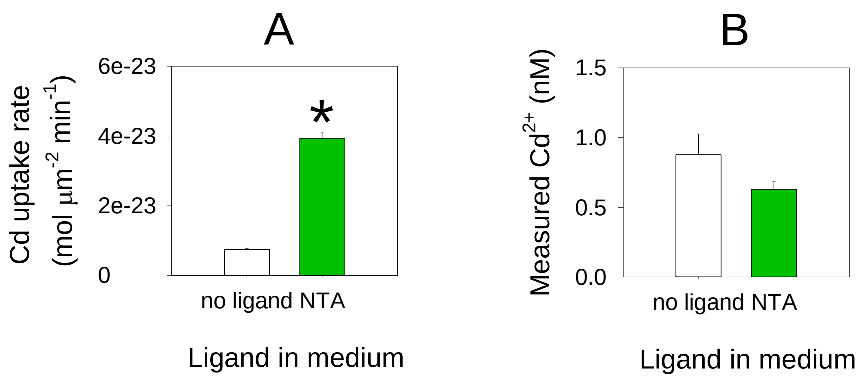

Figure 4. (A) Cd uptake rates (n = 3, mean ± SEM) at 1 nM bulk Cd^{2+} in the absence or presence of NTA by NH_4^+-acclimated *C. reinhardtii* (CPCC11) at 5.1-6.1 × 10^4 cells·mL^{-1}; and (B) the free Cd^{2+} concentration (n = 3, mean ± SD) measured by the ion-exchange technique in the 0.2-µm filtrates of the exposure solutions. The asterisk above the bar indicates a significant difference ($p < 0.05$). In the 'no ligand' treatment, the actual concentration of total Cd (i.e., 1.42 nM total Cd) was slightly higher than the theoretically required concentration (i.e., 1.21 nM total Cd) to make up for the small decrease (6%-15%) in total Cd concentration during the one hour uptake test. Based upon the measured concentration of total Cd in the filtrates, the calculated concentration of free Cd^{2+} in the 'no ligand' treatment (i.e., 0.96 nM Cd^{2+}) was also very close to that in the 'NTA' treatment (i.e., 1.07 nM Cd^{2+}).

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