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Can freshwater phytoplankton access cadmium bound to low-molecular-weight thiols?

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Abstract

Recent studies have suggested that low-molecular-weight (LMW) ligands can enhance trace metal uptake by marine diatoms. We have extended this work to freshwater phytoplankton and have investigated Cd bioavailability in the presence of LMW thiols, with two green algae (*Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata*) and a cyanobacterium (*Anabaena flos-aquae*). Using nitrilotriacetic acid (NTA) to hold the free Cd^{2+} concentration constant, we progressively added cysteine to the exposure medium and determined short-term Cd uptake rates for each organism; the Cd uptake rates remained constant and were unaffected even when the concentrations of the Cd-cysteine complexes exceeded that of the free Cd^{2+}. Similar results were obtained with algae pre-acclimated to different nitrogen sources or low zinc concentrations, even though such treatments affect their ability to utilize amino acids or take up Cd. A lack of effect was also observed for the two green algae with penicillamine, mercaptosuccinic acid, dithiothreitol and glutathione. These results suggest that the Cd-thiol complexes do not contribute to Cd uptake. However, when the experiments were repeated with cysteine in the absence of NTA, at the same Cd^{2+} concentration, a significantly higher Cd uptake rate was observed in the presence of the L- or D-cysteine stereoisomers than in the presence of NTA. This enhancement of Cd uptake in the presence of cysteine alone was sensitive to the nitrogen nutrition of these microorganisms, and we suggest that the enhancement likely results from biologically-induced chemical oxidation of cysteine in the boundary layer adjacent to the algal cell.

In natural waters, many trace metals tend to be largely bound by a variety of complexing agents, and numerous laboratory studies using synthetic ligands such as ethylenediaminetetraacetic acid (EDTA) or nitrilotriacetic acid (NTA) have demonstrated that complexation of a metal normally leads to a decrease in its bioavailability toward phytoplankton, by reducing the free metal ion concentration in the ambient medium (Sunda and Guillard 1976; Anderson and Morel 1978; Campbell 1995). These are the basic assumptions of the free ion activity model (FIAM) and its derivative, the biotic ligand model, and they underpin most modern work on the toxicity of dissolved metals (Paquin et al. 2002).

The aforementioned synthetic ligands are unassimilable, unlike many natural ligands, and thus experiments with them may not mimic the effect of naturally-occurring ligands on the bioavailability of metals to phytoplankton in "real-world" waters. For instance, natural ligands (e.g., thiosulfate, low-molecular-weight [LMW] organic metabolites) can be indirectly utilized or directly taken up by phytoplankton to satisfy their nutritional needs (Antia et al. 1991), and it has been suggested that metals bound to them can in some cases be accidentally transported into cells via membrane transporters (Campbell et al. 2002; Walsh et al. 2015). Moreover, several documented natural ligands released by certain groups of microorganisms have an exceptional capability to sequester metals, and these metal-ligand complexes (e.g., Fe-siderophores, Co-cobalamin, Cu-methanobactin) can be taken up directly by specific microorganisms for their physiological requirements (Vraspir and Butler 2009), especially when essential metals are otherwise scarce and insufficient. However, evidence for chlorophytes directly taking up these metal-ligand complexes is lacking.

Recently, several intriguing studies have postulated that metals bound to LMW ligands can be utilized by marine phytoplankton. For instance, higher zinc uptake rates in the presence of several thiols and amino acids (i.e., cysteine, glutathione, phytochelatin-2, and histidine) were observed for marine diatoms (Aristilde et al. 2012; Xu et al. 2012; Kim et al. 2015), and the enhancement was more dramatic in Zn-limited cells than in Zn-replete cells. Interestingly, it has also been observed that cysteine enhances Cu bioavailability to a

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Additional Supporting Information may be found in the online version of this article.

Cu-limited marine diatom (Walsh et al. 2015) and indigenous marine phytoplankton (Semeniuk et al. 2015). In earlier studies with freshwater algae, higher toxicity of Zn and Cd in the presence of alanine and citrate than in the presence of NTA was also reported (Errécalde et al. 1998; Errécalde and Campbell 2000). These studies suggest that under certain circumstances metals bound to natural LMW ligands may contribute to and enhance metal uptake by algal cells. However, the means by which algae access metals bound to LMW ligands remain unclear.

Natural ligands are a complex mixture of ill-defined compounds, including humic substances, microbial exudates, and biogenic molecules released by cell lysis. Exudates from actively growing phytoplankton are often dominated by a wide range of LMW compounds such as amino acids (Sarmento et al. 2013), and thiol compounds have been identified as a major component of the ligands exuded by the ubiquitous coccolithophore *Emiliania huxleyi* (Leal et al. 1999). Although thiols are known to be present in waters (Thomas 1997), these ligands are generally assumed to play a minor role in regulating metal speciation due to their low concentrations in bulk waters. However, recent results indicate that thiol concentrations may be up to three orders of magnitude higher in periphytic biofilms than in the overlying water column (Leclerc et al. 2015), suggesting that in microenvironments close to algal cells, thiols may in fact affect the speciation of some (soft) trace metals.

In this study, we investigated if dissolved Cd bound to LMW thiols can be accessed by two model freshwater algae and one cyanobacterium. The choice of Cd as the trace metal was guided by its emergence as an under-appreciated contaminant in freshwater environments, especially in the soft waters of the Canadian Shield and downwind from base metal smelters (Borgmann et al. 2004; Campbell and Gailer 2016). Cysteine was used as the model ligand and several other thiols were also studied. The methodology involved keeping the free Cd^{2+} concentration constant in the exposure medium by buffering the solution with NTA and then increasing the metal-thiol complex concentration by increasing the total thiol concentration (Xu et al., 2012); Cd uptake rates were determined in the presence and absence of the Cd-thiol complexes. This approach minimizes any interference from algal exudates and their potential influence on metal speciation in the exposure medium, and the conclusions are independent of the exact stability constants for the formation of the Cd-thiol complexes. It was hypothesized that the Cd-thiol complexes could directly contribute to the overall Cd uptake by (1) uptake of intact metal-thiol complexes via ligand transporters (Errécalde and Campbell 2000; Walsh et al. 2015) or (2) formation of a ternary complex with metal transporters (Aristilde et al. 2012). It was further hypothesized that if mechanism (1) were operative, the magnitude of the effect would change when the properties of the ligand transport systems were changed, whereas if mechanism (2) applied, the magnitude of the effect would change when the properties of the systems involved in transmembrane Cd transport were changed. Specifically, in this study, we modified the nutrient status of our microorganisms (nitrogen and zinc), since amino acid utilization (Munoz-Blanco et al. 1990; Palenik and Morel 1990; Antia et al. 1991) is known to be affected by the inorganic nitrogen source used by phytoplankton and pre-acclimation to Zn has been shown to affect the membrane transport of Cd in some algae (Price and Morel 1990; Xu et al. 2007; Lavoie et al. 2012*b*). Our results consistently showed that the Cd-thiol complexes did not contribute to the overall Cd uptake in presence of NTA but that they did in the absence of NTA, for these microorganisms that had been acclimated under specific nitrogen nutrition regimes.

Materials and methods

Phytoplankton culture

Aseptic strains of *Chlamydomonas reinhardtii* (CPCC11) (a wild strain, nitrate reductase-deficient, that cannot utilize nitrate for growth), *Pseudokirchneriella subcapitata* (CPCC37) and *Anabaena flos-aquae* (CPCC67) were obtained from the Canadian Phycological Culture Centre (CPCC) of the University of Waterloo (Ontario, Canada). The two green algae were grown in a modified high salt medium (MHSM-1, Supporting Information Table S1; Fortin and Campbell 2001), whereas the cyanobacterium *A. flos-aquae* was grown in BG-11 medium (Supporting Information Table S1; Andersen 2005). The microorganisms were grown in controlled environmental growth chambers (Conviron, CMP3023): for the two green algae, an illumination of 80–100 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 20°C and agitation at ~100–120 rpm; for *A. flos-aquae*, a day/night period 12/12 h at 18°C, a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and no agitation. The growth rates were 1.4 d^{-1} for *C. reinhardtii*, 1.0 d^{-1} for *P. subcapitata* and 0.15 d^{-1} for *A. flos-aquae*.

All culture media and acid-cleaned glassware were autoclaved at 121°C for 15 min prior to use, and the cultures were monitored periodically throughout the study by plating onto nutrient agar to ensure the absence of other living microbes such as bacteria. Unless otherwise indicated, all filtrations were performed with polycarbonate filters (Isopore™ membrane filters, Merck Millipore).

Modification of N nutrition for the algae

The procedures used to acclimate the algal cells to different nitrogen nutrition regimes are described below (Supporting Information Table S1).

***L-cysteine acclimated* C. reinhardtii.** Following 2–4 d growth in MHSM-1 medium, the cells were filtered (2 μm) in a sterilized filtration apparatus in a laminar flow hood and then resuspended in sterilized MHSM-1 medium (pre-autoclaved) with 5 mM L-cysteine (sterilized by 0.2-μm filtration) as the

only N source (Cys-MHSM-1). After a 2–4 d acclimation in the Cys-MHSM-1 medium, the cell population typically increased 1.8-fold. This slower than normal growth was likely due to the utilization of intracellular N reserves; a long-term culture test indicated that the Cys-MHSM-1 medium could not support the growth of *C. reinhardtii*.

***NH_4^+-acclimated* P. subcapitata.** The algae were grown in NH_4^+-MHSM-1 (i.e., NH_4^+ as the only N source) over 1 month before the uptake tests, with one to four transfers into fresh growth medium each month. The growth rate was 1.8 d^{-1}.

***NO_3^--acclimated* P. subcapitata.** The algae were grown in NO_3^--MHSM-1 (i.e., NO_3^- as the only N source) over 1 month before the uptake tests, with one to four transfers into fresh growth medium each month. The growth rate was 1.3 d^{-1}.

***NO_3^--acclimated or N_2-fixing* A. flos-aquae.** The green-blue alga grows very well in the BG-11 medium (i.e., NO_3^- as N source), whereas it grows very slowly with N_2 as its sole N source or does not grow at all with NH_4^+ as its sole N source under these growth conditions.

Modification of Zn nutrition for the two green algae

The algal cells were acclimated for 10–12 d in a low Zn MHSM-1 medium (i.e., at 9 pM Zn^{2+}; transferred into fresh low-Zn medium every 3 d or 4 d), whereas the Zn-replete cells (i.e., high Zn) were cultured in parallel in the MHSM-1 medium (i.e., at 910 pM [Zn^{2+}]) (Supporting Information Table S1). For the two algae, the growth rate of low Zn-acclimated cells was comparable to or even slightly higher than that of the high Zn-acclimated cells.

Exposure media and metal uptake

Preparation

All solutions were prepared under a laminar flow hood. The exposure medium for each species (*see* Supporting Information Note S1 and Supporting Information Table S1 for details) was a simplified version of the corresponding culture/acclimation medium; neither P nor trace metal-EDTA stock solutions were added to the exposure media, in order to better control Cd speciation and to avoid the possible effects of other trace metals on Cd uptake. Most chemicals (ACS grade or higher) were purchased from Sigma-Aldrich unless otherwise noted. Carrier free ^{109}Cd (initial specific activity 387 $Ci \cdot g^{-1}$ from Eckert and Ziegler and 492 $Ci \cdot g^{-1}$ from Perkin Elmer) was used to follow Cd uptake at low free Cd^{2+} concentrations (i.e., 0.1 nM, 0.2 nM, or 1 nM). The stable Cd (1 $mg \cdot L^{-1}$) stock solution was prepared by dilution of a 1 mg $Cd \cdot mL^{-1}$ standard solution (PlasmaCAL, SCP Science) in 0.5% HNO_3 (Optima, Fisher Sci.).

To ensure that no oxidation of cysteine occurred, tris(2-carboxyethyl)phosphine (TCEP, a reducing agent) was added or O_2-free water (i.e., pre-boiled water, cooled and flushed with 90% N_2, and 10% CO_2) was used in some tests. The thiol concentration in the cysteine solution (freshly prepared in Milli-Q water), as determined by the Ellman test, was exactly the same as the nominal value, indicating that the cysteine stock was not oxidized. Moreover, the Ellman test confirmed that there was no oxidation of cysteine in the exposure media within 1 d of preparation. The cysteine and NTA solutions were freshly prepared before the tests to minimize the danger of cysteine oxidation or other unknown chemical changes.

All stock solutions were filtered through 0.2 μm filters (Merck Millipore) prior to use, whereas the NaOH stock was filtered with Supor®-200 filters (0.2 μm, Pall Corporation). In exposure media including both NTA and thiols, thiols (ACROS Organics or Sigma-Aldrich) were first equilibrated with Cd in a small volume of water (with or without TCEP; final TCEP concentration $\geq$ thiol concentration) for 2–6 h and we then added the mixture to the NTA-buffered medium that had been pre-equilibrated for 24 h, and left the solution to equilibrate for an additional 5–24 h before the initiating the uptake tests. Exposure media including one single ligand were equilibrated for 24 h or overnight.

After the equilibration period, in some experiments, the final exposure solutions were re-filtered (0.2 μm) before initiation of the uptake tests (to confirm that no precipitation had occurred in the exposure medium). We collected small subsamples of the exposure media (1–2 mL) before adding the algal cells, to determine the ^{109}Cd radioactivity. 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 the uptake tests.

Calculation of Cd speciation

The speciation of Cd in the exposure medium was calculated with chemical equilibrium software (MINEQL + 4.62; Schecher and McAvoy 2001). Stability constants for the formation of metal-ligand complexes are shown in Supporting Information Table S2. The pH buffer (MOPS) does not complex Cd significantly (Soares et al. 1999) but the protonation constants for MOPS were added to the MINEQL database (Soares et al. 1999). Stability constants for TCEP as determined by Krezel et al. (2003) were also considered. Input data included the total concentrations of the major cations, anions, NTA, cysteine, and Cd. The pH was set at 7.0 unless otherwise noted and the simulations were run for solutions that were open to atmosphere. Precipitation of all solids was allowed. The experiments were designed such that the concentrations of all free metal ions (i.e., Cd^{2+}, Ca^{2+}, Mg^{2+}, Na^+, and K^+) and the ionic strength was kept constant for a given experiment, regardless of the ligands used.

Short-term Cd uptake

For a test with a given algal batch, a part of the cell culture was harvested on 2-μm filters, rinsed four times with a rinse solution (Supporting Information Note S1), and resuspended in a small volume (5–10 mL) of the rinse solution. Small subsamples (0.1 mL) were immediately removed and used for determinations of cell density and algal diameter with an electronic particle counter (Multisizer™ 3 Coulter

Counter® with a 70-μm aperture; Beckman, U.S.A.) after adding 9.9 mL Isoton III electrolyte (Beckman, U.S.A.). The cell densities in the exposure media were 0.5–1 $\times$ 10^5 cells·mL^{-1} for *C. reinhardtii*, 1–5 $\times$ 10^5 cells·mL^{-1} for *P. subcapitata*, and 1–1.6 $\times$ 10^5 cells·mL^{-1} for *A. flos-aquae*. The surface area was calculated with the assumption that individual algal cells are spherical.

The rinsed and resuspended cells were added to two to four replicate flasks containing the exposure medium (100–300 mL), and the flasks were then placed in the growth chambers under identical growth conditions. During a 1-h exposure period, we collected algal samples one to five times by removing samples (50 mL or 100 mL solution) from each flask at each time point. The algal cells were filtered onto two superimposed 2-μm filters, soaked for 10 min with 10 mL rinse solution containing 0.1 mM or 1 mM EDTA (Supporting Information Note S1 for details), and finally rinsed two to three times with 10 mL of the EDTA solution. The rinsed filters were placed in glass vials and covered with water. We also collected filtered water samples at each time point in one experiment for each alga, just after collection of the algae by filtration and before washing them with the EDTA solution, in order to confirm the constancy of the total Cd concentration during the uptake period.

As mentioned above, there were two to four replicates for a single experiment with a given algal batch. In addition, some of the tests were repeated with different algal batches to ascertain the reproducibility of the results. Appreciable variations in the absolute Cd uptake rates at a given Cd^{2+} concentration were observed from one algal batch to another (Supporting Information Table S3), for reasons that were not explored, and thus all experiments designed to identify treatment effects were run with the same algal batch.

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): counting window (Dynamic) peak position, 22 keV; low boundary, 16 keV; high boundary, 32 keV; counting spectrum type, single peak. To ensure constant sample geometry, the samples from a given experiment were placed in glass counting vials filled with the same volume of water (i.e., 2 mL or 5 mL). Each sample was counted for 200 s and the detected signals of the algal samples were considered to be acceptable when higher than 50 counts per minute (cpm).

Cadmium accumulation by the algae was calculated from the measured radioactivity in the algae and the known specific radioactivity of Cd in the exposure solution. Ratios of ^{109}Cd to total Cd in exposure solutions were lower than 1%, and thus the addition of trace ^{109}Cd did not change the total Cd concentration. 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.

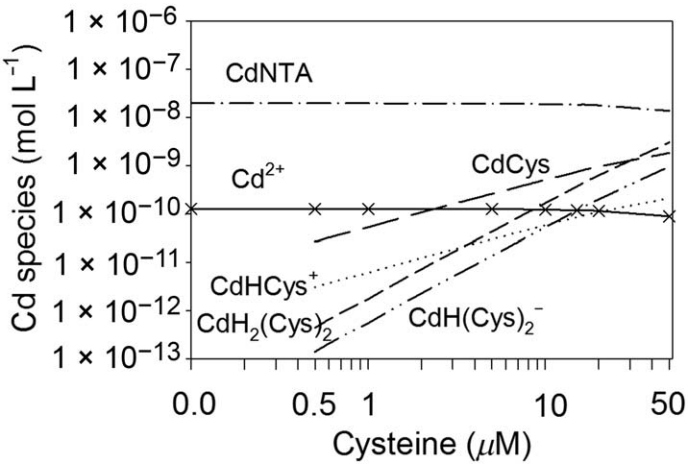

Fig. 1. Calculated concentrations of various chemical forms of Cd as a function of the cysteine concentration. The symbols ($\times$) indicate at which concentrations the Cd uptake was determined in this study. In solution, total Cd = 20 nM, total NTA = 10 μM, total Cys = 0–50 μM, and pH = 7.0 (*See* Supporting Information Note S1 and Supporting Information Table S1 for the medium composition).

Algal uptake of Cd is expressed as an internalization flux, which was calculated from the linear regression of Cd internalized by the algal cells over time and has been normalized on the basis of the cell surface area (mol Cd·μm^{-2}·min^{-1}). Internalized Cd refers to the metal associated with cells after washing with EDTA (Hassler et al. 2004). Cadmium uptake rates were linear over a 1-h exposure period under all the tested culture conditions (e.g., Supporting Information Fig. S1). In some tests (particularly in the presence of cysteine alone), the linear regressions did not pass through the origin, and in such cases we calculated the uptake rate from at least two time points.

Statistical analyses

Data analyses were performed with two software packages: SPSS (Version 16.0) and SigmaPlot 12.5 for Windows. We compared the Cd uptake rates between or among treatments for the same algal batch by using a *t*-test for two treatments, the univariate analysis of variance (one-way ANOVA) followed by a Student-Newman-Keuls post hoc test for more than two treatments, or a general linear model for comparing the slopes (i.e., the linear Cd uptake rates) (SPSS 16.0). Linear regressions for the 1-h Cd uptake rates were calculated with SigmaPlot. The significance level for all tests was set at $p < 0.05$, unless otherwise indicated.

Results

Free Cd^{2+} and Cd-thiol complexes in solution

To minimize any interference from algal exudates and their potential influence on metal speciation in the exposure medium, NTA was used as a metal buffer to ensure that both the total Cd concentration and more importantly the free

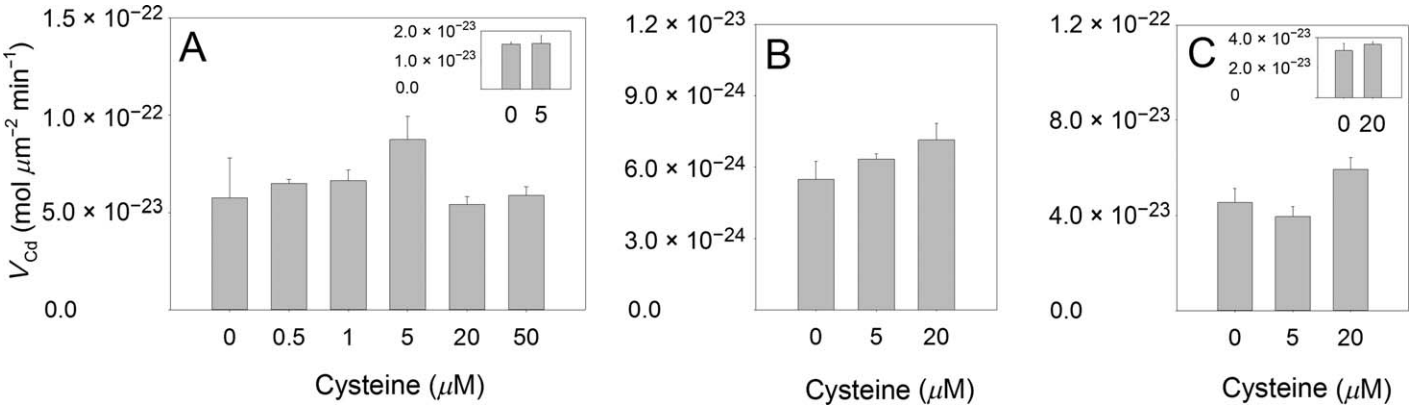

Fig. 2. One-hour Cd uptake rate (mean ± SD, $n = 3$–4) at 0.1 nM Cd^{2+} (buffered by NTA) in the presence or absence of L-cysteine by (**A**) *C. reinhardtii* (CPCC11) at 1×10^5 cells mL^{-1}; (**B**) *P. subcapitata* (CPCC37) at 1×10^5 cells·mL^{-1}; (**C**) *A. flos-aquae* (CPCC67) at 1×10^5 cells·mL^{-1}. Data for each panel were from a single test (i.e., the same algal batch), and inserts in panels (**A**) and (**C**) show one independent repeated test (which confirmed that the small variation in the tests involving *C. reinhardtii* at 5 μM total cysteine and *A. flos-aquae* at 20 μM total cysteine was due to experimental variation). TCEP was added to avoid any oxidation of cysteine in the bulk water. The two green algae were cultured in the MHSM-1 medium whereas the cyanobacterium was cultured in BG-11 medium.

Cd^{2+} concentration would remain constant during the 1-h exposure period. Radioactivity measurements on the filtrates confirmed that the total Cd concentration remained constant in the presence of NTA.

Since the ratio of total Cd to free Cd^{2+} was very high (e.g., 200 : 1), the progressive addition of thiols to the NTA-buffered system allowed us to increase the concentrations of the Cd-thiol complexes without appreciably changing [Cd^{2+}] (Fig. 1). In the Cd-cysteine-NTA system, with a free Cd^{2+} concentration buffered at 0.1 nM and for total cysteine concentrations less than 20 μM, the major Cd-thiol complex was the electrically neutral Cd-cys species; only at cysteine concentrations above ~20 μM did the $Cd(cys)_2$ complexes become quantitatively important. For total cysteine concentrations greater than 2 μM, the total concentration of Cd-cys complexes was higher than that of free Cd^{2+}. Similarly, in the experiments with the other thiols (i.e., penicillamine, glutathione, dithiothreitol [DTT] and mercaptosuccinic acid [MSA]), the range of thiol concentrations was also chosen to ensure that the concentration of the Cd-thiol complexes exceeded that of the free Cd^{2+} ion.

Uptake of Cd by nutrient-sufficient algae in the absence or presence of cysteine

Unlike the results reported in the literature for marine diatoms (*see* Introduction), short-term uptake of Cd (0.1 nM Cd^{2+}, buffered by NTA) by *C. reinhardtii* was unaffected by the presence of cysteine in the range 0.5–50 μM total cysteine (Fig. 2A), despite the notable increase in the concentration of Cd-cysteine complexes that occurs over this range (cf. Fig. 1). We did not observe any significant influence of TCEP addition (a reducing agent) on algal uptake of Cd in the presence/absence of cysteine (Supporting Information Fig. S2). A similar lack of effect of cysteine addition was found for *P. subcapitata* and *A. flos-aquae* (Fig. 2B,C). Note that these cells were grown in and harvested from growth media with sufficient major and minor nutrients.

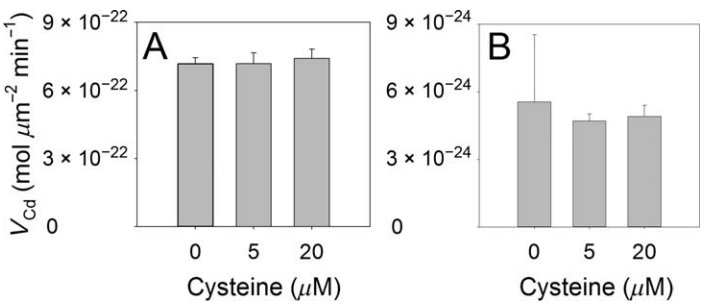

Fig. 3. One-hour Cd uptake rate (mean ± SD, $n = 4$) at 0.1 nM Cd^{2+} (buffered by NTA) in the presence or absence of L-cysteine by (**A**) low Zn acclimated *C. reinhardtii* (CPCC11) at 1×10^5 cells·mL^{-1} and (**B**) low Zn acclimated *P. subcapitata* (CPCC37) at 1×10^5 cells·mL^{-1}. Both algae were pre-acclimated in the low-Zn MHSM-1 medium (i.e., 9 pM free Zn^{2+}). Data for each panel were from a single test (i.e., the same algal batch). TCEP was added to avoid any oxidation of cysteine in the bulk water.

Influence of acclimation (low Zn or different N source) on algal uptake of Cd in the absence or presence of cysteine

Since experiments with marine algae had shown that cysteine only significantly enhanced zinc uptake if the alga had been grown under Zn-limiting conditions (Aristilde et al. 2012), we tested the effects of Zn limitation on our two freshwater chlorophytes. Both *C. reinhardtii* and *P. subcapitata* were acclimated in the low-Zn MHSM-1 medium (9 pM Zn^{2+}; MINEQL+ simulations) and then exposed to 0.1 M free Cd^{2+} in NTA-buffered solutions in the presence of 0 μM, 5 μM, and 20 μM cysteine. Uptake of Cd by the *C. reinhardtii*

cells that had been acclimated to low-Zn growth conditions was higher than for the cells grown in MHSM-1 medium (Supporting Information Fig. S3A), indicating that the acclimation step had been effective in enhancing Cd (and Zn) uptake; a similar observation was reported for this alga by Lavoie et al. (2012*a*). On the other hand, the modification in Zn nutrition did not affect Cd uptake by *P. subcapitata* (Supporting Information Fig. S3B). Most importantly, in neither case did acclimation to low-Zn conditions lead to an enhancement of Cd uptake in the presence of cysteine (Fig. 3A,B).

We further tested the hypothesis that the bioavailability of Cd-cysteine complexes is associated with cysteine utilization. Previous work has shown that the ability of unicellular algae to utilize amino acids can be affected by inorganic nitrogen forms and concentrations that are present in the growth culture (Antia et al., 1991). However, for *P. subcapitata* cells that had been grown in the presence of either NH_4^+ or NO_3^- as nitrogen sources, Cd uptake was unaffected by the presence of 20 μM cysteine (Fig. 4A,B).

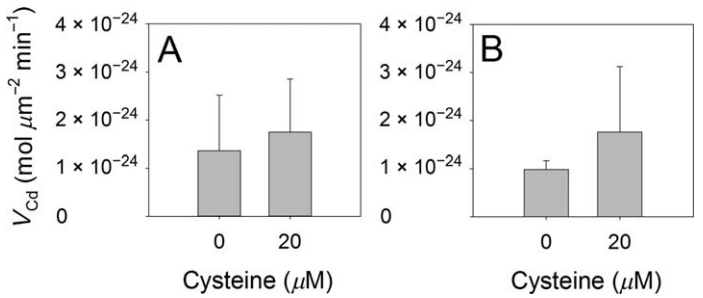

Fig. 4. One-hour Cd uptake rate (mean ± SD, $n = 2$–3) at 0.1 nM Cd^{2+} (buffered by NTA) in the presence or absence of L-cysteine by (**A**) NH_4^+-acclimated *P. subcapitata* (CPCC37) at 1.5–1.6 × 10^5 cells·mL^{-1} and (**B**) NO_3^--acclimated *P. subcapitata* (CPCC37) at 1.5–1.6 × 10^5 cells·mL^{-1}. Data for each panel were from a single test (i.e., the same algal batch).

Uptake of Cd by nutrient-sufficient algae in the absence or presence of other LMW thiols

All the preceding experiments were carried out in the presence of L-cysteine as the model thiol, and all the data consistently show that the addition of cysteine did not affect Cd uptake when NTA was present in the medium as a metal buffer. Applying the same experimental protocol, we tested the effect of other LMW thiols (L-penicillamine [Pen], glutathione [GSH], DTT, and MSA) on Cd uptake. In these experiments, a single thiol concentration was chosen such that the Cd-thiol complexes were always present at concentrations greater than the free Cd^{2+} concentration (0.2 nM Cd^{2+}; MINEQL+ simulations). Again, in no case did the presence of the thiol lead to enhanced Cd uptake by the two green algae (Fig. 5, Supporting Information Fig. S4), despite the presence of Cd-thiol complexes.

Uptake of Cd by algae in the presence of cysteine but the absence of NTA

In our final experiments, we adopted a different experimental protocol, in which the algae were exposed to the same free Cd^{2+} concentration (either 0.1 nM or 1 nM) in the presence of NTA or in the presence of cysteine. As predicted by the FIAM, we observed that the uptake rates were identical (NTA- vs. Cys-buffered media) for nutrient-sufficient *C. reinhardtii* (i.e., grown in MHSM-1 medium) (Fig. 6A). In this figure, note that at the same total Cd concentration (20 nM), the highest uptake rate is observed in the absence of any metal-binding ligand; addition of either NTA or cysteine, at concentrations needed to reduce the free Cd^{2+} concentration to 1 nM, reduced the uptake rate to identical values. Similarly, at free Cd^{2+} concentrations of 0.1 nM, buffered with either NTA or cysteine, Cd uptake rates were identical for N_2-fixing *A. flos-aquae* (Supporting Information Fig. S5).

To our surprise, for L-cysteine acclimated *C. reinhardtii*, the Cd uptake rate in the presence of cysteine alone was higher (2.3×) than in the presence of NTA alone (Fig. 6B,

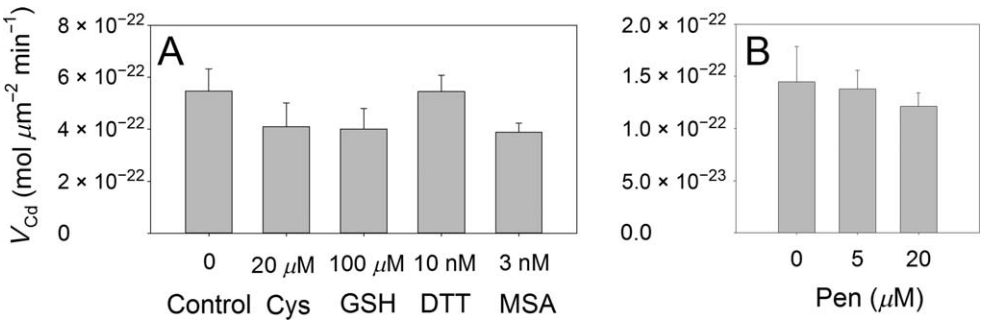

Fig. 5. One-hour Cd uptake rate (mean ± SD, $n = 3$) at 0.2 nM Cd^{2+} (buffered by NTA) in the presence or absence of: (**A**) L-cysteine (Cys), glutathione (GSH), DTT, MSA and (**B**) L-penicillamine (Pen), by *C. reinhardtii* (CPCC11) at 7.7–8.6 × 10^4 cells·mL^{-1}. In solution, total [Cd] = 20 nM, [NTA] = 10 μM, pH = 6.85, and [Cd-thiol complexes] > [Cd^{2+}] as calculated with MINEQL+. The solutions were prepared in boiled and cooled Milli-Q water that was flushed with N_2/CO_2 (10% CO_2) to minimize any oxidation of the thiols. The algae were cultured in MHSM-1 medium (i.e., with NH_4^+ as the nitrogen source). Data for each panel were from a single test (i.e., the same algal batch).

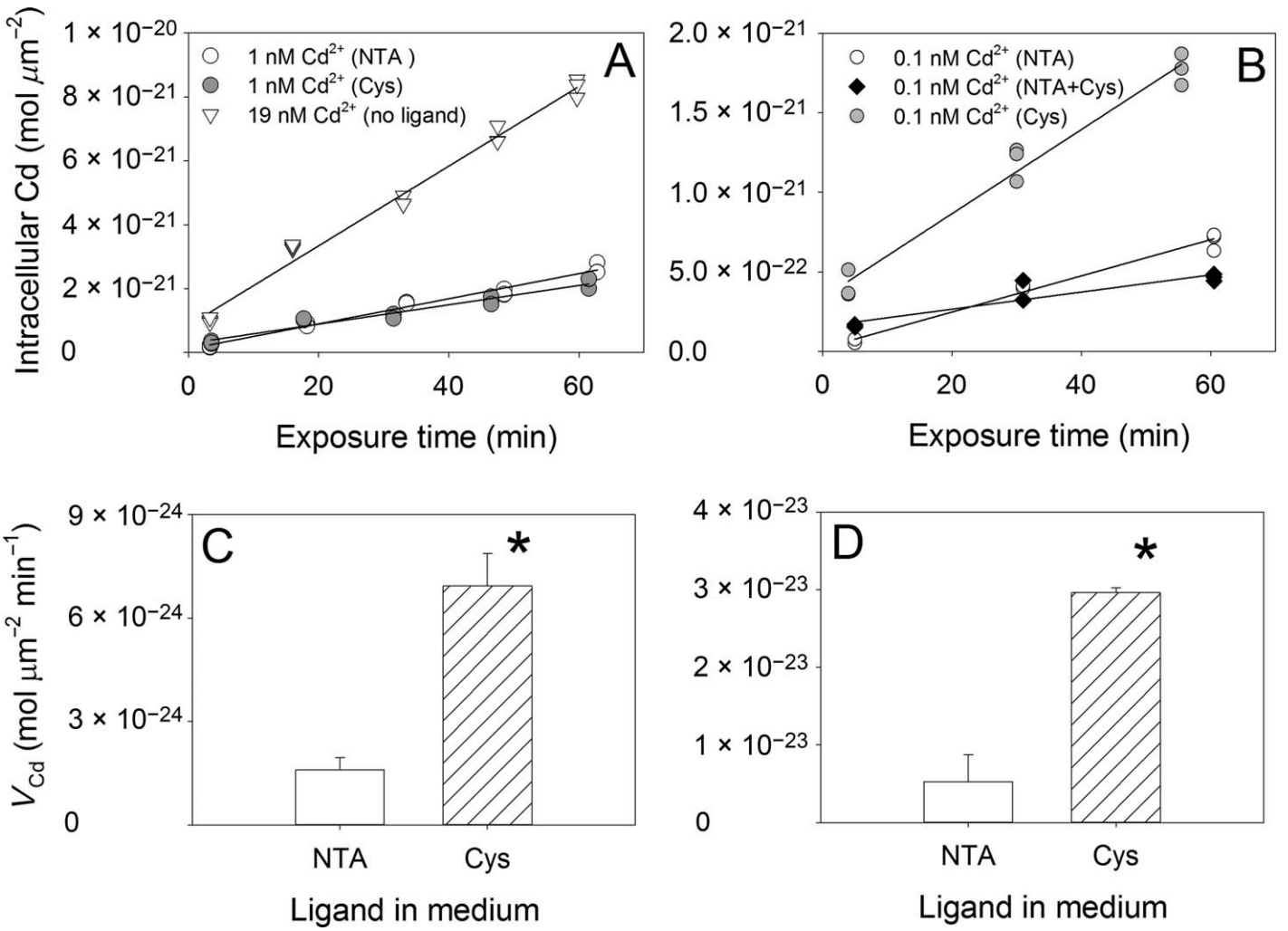

Fig. 6. One-hour Cd uptake rate (mean ± SD, $n = 2$–3) at the same Cd^{2+} concentration in the presence of either NTA or L-cysteine alone by (**A**) MHSM-1 acclimated *C. reinhardtii* (CPCC11) at 5–8 × 10^4 cells·mL^{-1}; (**B**) L-cysteine acclimated *C. reinhardtii* (CPCC11) at 9.3–10 × 10^4 cells·mL^{-1}; (**C**) NO_3^--acclimated *P. subcapitata* (CPCC37) at 1.5–1.6 × 10^5 cells·mL^{-1} and (**D**) NO_3^--acclimated *A. flos-aquae* (CPCC67) at 1.5 × 10^5 cells·mL^{-1}. Data for each panel were from a single experiment (i.e., the same algal batch) and the controls are the treatments with addition of NTA as the metal buffer. In all solutions, total Cd = 20 nM and pH = 7.0. In the exposure solution for panel (**A**), ligand = 1.09 μM NTA or 20 μM Cys (i.e., 1 nM Cd^{2+}); note that panel (**A**) also includes a plot for the same total Cd concentration as the other two plots but with neither NTA nor cysteine present to complex the Cd (i.e., 19 nM Cd^{2+}). In the solutions for panels (**B**), (**C**), and (**D**), ligand = 10 μM NTA or 75 μM Cys (i.e., 0.1 nM Cd^{2+}). An asterisk (*) above the bars indicates a significant difference ($p < 0.05$).

general linear model, $p < 0.001$). Moreover, for *P. subcapitata* acclimated to grow with nitrate as the nitrogen source (Fig. 6C) and for *A. flos-aquae* grown in BG-11 medium (Fig. 6D), this rate enhancement in the presence of L-cysteine was more marked (4.4× for the chlorophyte, and 5.6× for the cyanophyte, $p < 0.01$). When using the D-stereoisomer of cysteine as the metal buffer, an enhancement of the same magnitude was observed for the L-cysteine acclimated *C. reinhardtii* (Supporting Information Fig. S6) and the nitrate-acclimated *P. subcapitata* (Supporting Information Fig. S7).

More interestingly, when the L-cysteine acclimated *C. reinhardtii* were re-supplied with NH_4^+ and cultured for 40 h, the enhancement of Cd uptake in the presence of cysteine disappeared (Supporting Information Fig. S8). On the other hand, when exposing NH_4^+-acclimated *C. reinhardtii* to the 0.2 μm-filtrate from an experiment with L-cysteine-acclimated cells that had demonstrated enhanced uptake in the presence of cysteine, the NH_4^+-acclimated cells showed no enhancement in Cd uptake (Supporting Information Fig. S9).

Discussion

Our results consistently show that our freshwater algae and cyanobacterium could not access the Cd bound to LMW thiols when NTA was present, an observation that differs from those reported for marine diatoms. However, we

observed that Cd bioavailability for a fixed free Cd^{2+} concentration in the presence of cysteine alone was higher than expected (i.e., higher than that the same Cd^{2+} concentration in the presence of NTA alone) for all the tested microorganisms except for NH_4^+-acclimated *C. reinhardtii* and N_2-fixing *A. flos-aquae*. As discussed below, our observations cannot be explained by diffusion limitation, by the uptake of intact metal complexes or by the formation of ternary surface complexes. We propose another mechanism—a "boundary layer effect"—to explain the contrast between our observations in freshwater systems and those in seawater.

No diffusion limitation

When considering exceptions to the FIAM, such as those that have been documented in the case of some LMW thiols and marine phytoplankton and in the present experiments in media that do not contain NTA, it is useful first to rule out the possibility of diffusive limitation. Specifically, if metal uptake is very rapid but the ambient concentration of the free metal ion is relatively low, diffusion of the free metal ion from the bulk solution to the microorganism surface may become the rate-limiting step in metal uptake. Under such conditions, dissociation of labile metal-ligand complexes near cell algal surface would increase the overall metal uptake rate (van Leeuwen 1999; Fortin and Campbell 2000). In the present case, for a bulk solution concentration of 0.1 nM Cd^{2+}, the maximum diffusive flux toward the cells, $J_{\text{diff max}}$, is $\sim 2.0 \times 10^{-21}$ mol μm^{-2} min^{-1} (*see* Supporting Information Note S2 for the calculation), more than 40-times higher than the measured Cd uptake rates in the presence of cysteine alone. Clearly, diffusive limitation is not operative in the present experiments.

Weak ligands promote metal uptake by marine phytoplankton but not by freshwater species

Based on the results published for marine diatoms only, we originally anticipated that Cd uptake would be enhanced in the presence of one or more thiols, as a result either of direct uptake of Cd-thiol complexes via membrane-bound permeases or of the formation of a ternary surface complex under conditions of local disequilibrium.

Uptake of intact metal-ligand complexes

With respect to the first possibility, evidence as to the ability of (marine) algae to assimilate intact metal-LMW complexes is uncertain. Aristilde et al. (2012) reported that additions of L- and D-stereoisomers of cysteine resulted in similar increases in Zn uptake rates in the presence of the strong chelator EDTA, ruling out uptake by a stereospecific Zn-Cys membrane transporter. However, Kim et al. (2015) suggested that Zn bound as the 1 : 1 Zn-histidine complex, but not as the 1 : 2 Zn-histidine complex, is taken up by the marine diatom *Thalassiosira weissflogii*, based upon a quantitative correspondence among the Zn uptake rate, equilibrium calculations and the polarographic measurement of Zn electroreactivity. Walsh et al. (2015) suggested two parallel mechanisms for the enhanced Cu uptake by Cu-limited *E. huxleyi* that was observed in the presence of D- and L-cysteine. The first mechanism involved the chemical reduction of Cu(II) to Cu(I) in the presence of cysteine, followed by the rapid uptake of Cu(I) by a high-affinity Cu(I) membrane transporter; both D- and L-cysteine could participate in such an uptake route, but clearly this cannot apply to redox-insensitive Cd(II). Walsh et al. (2015) also found evidence for a second mechanism, involving the uptake of an intact cysteine-Cu(I) complex, but this would only be operable in the case of the L-stereoisomer.

In the present case, for all five thiols tested (L-penicillamine [Pen], L-cysteine [Cys], glutathione [GSH], DTT, and MSA) and for the three test microorganisms, no enhancement of Cd uptake could be detected in media containing NTA as a metal buffer, even under conditions where the ambient concentrations of the Cd-thiol complexes exceeded that of the free Cd^{2+} ion. Since the ability of some algae to take up or indirectly utilize amino acids for their nitrogen nutrition is enhanced when ambient ammonium concentrations are low (Munoz-Blanco et al. 1990; Palenik and Morel 1990; Antia et al. 1991), we repeated these experiments with cysteine as the model thiol and algal cells that had been cultured in the absence of NH_4^+. Despite this pre-treatment, Cd uptake by nitrate-acclimated *P. subcapitata* (Fig. 4B) and L-cysteine-acclimated *C. reinhardtii* (Fig. 6B) was not enhanced in the presence of L-cysteine when NTA was also present. We conclude that uptake of intact metal-LMW thiol complexes does not occur with our algae under our experimental conditions, and it cannot explain the enhanced uptake of Cd in the experiments run in the absence of NTA (Fig. 6B–D; Supporting Information Figs. S6, S7, S9A, S10).

Involvement of ternary complexes at the algal surface

In theory, if the rate-limiting step in metal uptake is transmembrane transport (i.e., if the algal surface is in equilibrium with the ambient solution), the formation of a ternary complex (e.g., cell-X-Cd-cys) will not affect the metal uptake rate (Morel 1983).

$$\text{cell-X} + \text{Cd} \rightleftarrows \text{cell-X-Cd} \xrightarrow{\text{slow}} \text{X-cell(Cd)}$$

$$\text{cell-X} + \text{Cd} + \text{cys} \rightleftarrows \text{cell-X-Cd-cys} \xrightarrow{\text{slow}} \text{X-cell(Cd)} + \text{cys}$$

On the other hand, if local disequilibrium conditions exist, such that the cell-X-Cd and cell-X-Cd-cys surface complexes do not equilibrate, then it is possible that the ternary complex could participate in and enhance overall Cd uptake (F. M. M. Morel pers. comm.). This hypothesis was first advanced by Xu et al. (2012) and Aristilde et al. (2012), who suggested that ternary complexes could contribute to Zn uptake in Zn-deficient *T. weissflogii* and *E. huxleyi*.

However, our present data do not support this scenario, given the absence of enhanced Cd uptake in the presence of both NTA and cysteine for three nutrient-replete freshwater phytoplankton species and for two low-Zn acclimated green algae. Unlike the case for marine diatoms (Price and Morel 1990), there is no documented biological function of Cd in freshwater phytoplankton; it is possible that even under low-Zn conditions, freshwater algae have no "incentive for" and cannot effectively access Cd from Cd-LMW thiol complexes.

Why does addition of cysteine only lead to enhanced uptake of Cd in the absence of NTA (and with algae that have been acclimated under specific nitrogen nutrition conditions)?

In this study, enhanced uptake of Cd in the presence of cysteine alone was observed in the two algae and one cyanobacterium, and the enhancement was sensitive to the nitrogen source for these microorganisms. For instance, unlike the L-cysteine acclimated *C. reinhardtii* (Fig. 6B), no enhancement was observed if the cells had been cultured in the presence of NH_4^+ as a nitrogen source (Fig. 6A). Moreover, if the L-cysteine acclimated cells were re-supplied with NH_4^+ and cultured for 40 h, the enhancement of Cd uptake in the presence of cysteine disappeared (Supporting Information Fig. S8). Similarly, the enhancement in Cd uptake by *A. flos-aquae* also depended upon the nitrogen forms used for its growth; the effect was observable in the presence of nitrate (Fig. 6D) but disappeared in the presence of N_2 alone (Supporting Information Fig. S5). We found a consistent enhancement of Cd uptake by various nitrogen-acclimated cultures of *P. subcapitata*, and the magnitude of the enhancement varied from one nitrogen form to another (Supporting Information Fig. S10).

Given that we could find no evidence for biological uptake of intact Cd-cysteine complexes by our microorganisms, we have focused on extracellular phenomena in the following discussion. For the experiments run with cysteine as the metal buffer, i.e., in the absence of NTA, measurements of the total cysteine concentration in the exposure medium showed no decrease over the period between the preparation of the medium and the end of the exposure (i.e., no inadvertent loss of cysteine and no increase in $[Cd^{2+}]$). Moreover, confirmation that differences in the bulk Cd^{2+} concentration were not responsible for the observed effects was obtained by exposing NH_4^+-acclimated *C. reinhardtii* to the 0.2 μm-filtrate from an experiment with L-cysteine-acclimated cells that had demonstrated enhanced uptake in the presence of cysteine (Supporting Information Fig. S9A); the NH_4^+-acclimated cells showed no enhancement when exposed to the filtrate (Supporting Information Fig. S9B).

In the absence of changes in the bulk solution chemistry, we considered the chemistry of the boundary layer next to the algal cells, which is immediately adjacent to the phytoplankton cell membrane and within the diffusion boundary layer ($\approx$ 10 μm; Wilkinson and Buffle 2004). The physicochemical conditions in the boundary layer have been reported to be different from those in the surrounding waters (Kuhn and Raven 2008; Milligan et al. 2009), and chemical speciation of trace elements might thus be different from that in the bulk solution. To explain the observation of enhanced Cd uptake in the presence of cysteine alone, and the disappearance of this effect in NTA-buffered systems, we suggest that the free Cd^{2+} concentration is higher in the boundary layer next to the algal cells than in bulk solution under certain experimental/physiological conditions, due to an alga-mediated breakdown or dissociation of the Cd-cys complexes.

The postulated breakdown of Cd-cys complexes cannot be an enzymatic reaction (e.g., ligand oxidation by membrane enzymes, including deamination enzymes), because a similar enhancement was also observed for L-cysteine-acclimated *C. reinhardtii* in the presence of D-cysteine, or in the presence of a high concentration of NH_4^+ added to the exposure solution before the 1-h uptake test (Supporting Information Fig. S6). A significantly higher Cd uptake rate was also observed for nitrate-acclimated *P. subcapitata* in the presence of D-cysteine than in the presence of NTA (Supporting Information Fig. S7). It is generally considered that phytoplankton cells cannot enzymatically oxidize D-amino acids and that a high ambient concentration of NH_4^+ inhibits the deamination of amino acids (Munoz-Blanco et al. 1990; Palenik and Morel 1990; Antia et al. 1991).

However, the enhancement may be due to a biologically-induced chemical reaction such as extracellular redox reactions involving algal metabolites (e.g., reactive oxygen/nitrogen species). Algae constantly release such species into the boundary layer, and the highest concentration of reactive oxygen/nitrogen species would be near the cell surface. The proposed extracellular reaction must be very fast, because the enhanced uptake was observed within the first 30 min (Supporting Information Fig. S6). Surprisingly, the postulated reaction is unaffected by the presence of the TCEP reducing agent; the enhancement in the presence of cysteine and TCEP was similar to that without addition of TCEP (Supporting Information Fig. S6).

Extracellular production of reactive oxygen/nitrogen species has been shown to be associated with nitrogen nutrition in algae/plants (Palenik and Morel 1990; Wilson et al. 2008). The absence of a cysteine enhancement of Cd uptake by the NH_4^+-acclimated *C. reinhardtii* may be associated with the dysfunction of nitrate reductase in this algal strain. Nitrate reductase plays a critical role in the intracellular metabolism of nicotinamide adenine dinucleotide, which regulates the production of reactive oxygen/nitrogen species (Solomonson and Barber 1990; Diaz et al. 2013). Note also that interrelationships among nitrogen nutrition, extracellular production of reactive oxygen/nitrogen species and the postulated ligand oxidation in the boundary layer appear to be species-

specific. For example, with the other green alga, *P. subcapitata*, the enhancement of Cd uptake was observed for all the tested nitrogen nutrition conditions (NH_4^+ alone, NO_3^- alone, L-cysteine alone, N-starvation, and a mixture of NH_4^+ and NO_3^-; Supporting Information Fig. S10), provided that NTA was not present in the exposure medium, whereas for *A. flos-aquae* cysteine-enhanced Cd uptake was observed for nitrate-acclimated *A. flos-aquae* but not for long-term N_2-acclimated cells (Supporting Information Fig. S5).

Our hypothesis offers an explanation for the apparent discrepancy between our observations in freshwater systems and those in seawater. In other words, the "boundary layer effect" could explain the weak-ligand enhancement of metal uptake by marine algae as well. Specifically, we suggest that extracellular oxidation and/or deamination of amino acids (cysteine and histidine) may contribute to the enhanced metal uptake observed in marine algae in the presence of amino acids. The experiments reported in the literature (Aristilde et al. 2012; Xu et al. 2012; Walsh et al. 2015) were performed in EDTA-buffered media with nitrate-acclimated diatoms, and deamination is observed in nitrate-grown marine phytoplankton (Palenik and Morel 1990). In seawater, EDTA is largely bound to Ca and Mg, and any free metal ions released from metal-LMW complexes in the boundary layer would not be immediately complexed by the EDTA; the kinetics of trace metal exchanges with EDTA in seawater are known to be slow (Hering and Morel 1989). Instead, some of the free metal ions released from the metal-LMW complexes could be immediately taken up by algal cells, given that the breakdown reactions are postulated to occur near the cell surface. In our freshwater medium, the majority of NTA is not complexed by any metal ion, and thus the free and protonated NTA can immediately sequester any free metal ion released from metal-LMW complexes in the boundary layer, thus suppressing any changes in metal speciation that could lead to an enhancement of metal uptake.

Implications

Our data confirm that intact complexes of Cd bound to LMW thiols are not assimilated by the tested algae and cyanobacterium, and they suggest that Cd bioavailability is determined by the concentration of free Cd^{2+} in the boundary layer, which can be different from that in the surrounding waters and might be regulated by the metabolism of these microorganism. A corollary of our "boundary layer effect" would be that ligand properties other than just their ability to bind metals might affect metal bioavailability. For example, metal bioavailability might vary in waters containing different types of extracellular ligands (e.g., redox-sensitive ligands, redox-insensitive ligands, or a combination of both types) at a fixed bulk free M^{z+} concentration. In addition, our results illustrate that the physiological conditions of the phytoplankton (e.g., their nitrogen source) may also play an important role in determining metal bioavailability. We suspect that uptake of other trace metals (e.g., Cu, Zn, Hg, etc.) by phytoplankton will also be sensitive to the proposed "boundary layer effect."

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Conflict of Interest

None declared.

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