# Why does cysteine enhance metal uptake by phytoplankton in seawater but not in fresh water?

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Short title: ambient Ca regulates bioavailability of Cd-cysteine complexes

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28 Abstract:

29 Low-molecular-weight weak ligands such as cysteine have been shown to enhance metal
30 uptake by marine phytoplankton in the presence of strong ligands, but the effect is not
31 observed in freshwater. We hypothesized that these contrasting results might be caused by
32 local cysteine degradation and a Ca effect on metal-ligand exchange kinetics in the boundary
33 layer surrounding the algal cells; newly liberated free metal ions cannot be immediately
34 complexed in seawater by Ca-bound strong ligands, but can be rapidly complexed by free
35 ligands at low Ca levels. The present results consistently support this hypothesis. At constant
36 bulk $Cd^{2+}$ concentrations, buffered by strong ligands: 1) at 50 mM Ca, cysteine addition
37 significantly enhanced Cd uptake in high-Ca pre-acclimated euryhaline *Chlamydomonas*
38 *reinhardtii* (cultured with cysteine as a nitrogen source to enhance local $Cd^{2+}$ liberation via
39 cysteine degradation); 2) at 0.07 mM Ca, this enhancement was not observed in the algae; 3)
40 at 50 mM Ca, the enhancement disappeared when *C. reinhardtii* were cultured with
41 ammonium (to inhibit cysteine degradation and local $Cd^{2+}$ liberation); and 4) cysteine addition
42 did not enhance Cd uptake by cysteine-cultured marine *Thalassiosira weissflogii* when the
43 concentration of immediately reacting strong ligands was sufficient to complex local $Cd^{2+}$
44 liberation.

45

46 Key words: trace metals, bioavailability, phytoplankton, phycosphere

47 **Introduction**

48 In both marine and fresh waters, trace metals are bound more or less strongly by organic
49 ligands;[1, 2] for instance, Fe is dominantly complexed by poorly defined organic ligands in
50 oceans[3] whereas a large fraction of Zn and Cd can be found as the free ions in some
51 freshwater bodies.[4] Metal complexation has long been implicated as a dominant control on
52 the bioavailability of trace metals to aquatic organisms, the assumption being that complexed
53 metals may act as a metal buffer or reservoir. Indeed, numerous laboratory studies with
54 synthetic strong ligands such as ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic
55 acid (NTA) have shown that the such hydrophilic metal-ligand complexes cannot be taken up
56 by phytoplankton;[5] in the presence of such organic ligands, metal uptake is best predicted by
57 the free metal ion concentration in the exposure medium.[6, 7]
58
59 Interestingly, recent studies with model and indigenous marine phytoplankton have reported
60 that in the presence of natural low-molecular-weight (LMW) ligands such as cysteine, histidine
61 and glutathione, uptake of Zn, Cu and Cd in certain treatments of algae cultures was always
62 greater than what would have been predicted on the basis of the free metal ion
63 concentrations, which were well buffered by strong ligands such as EDTA or *in situ* ligands.[8-11]
64 We refer to this design as a 'two-ligand' system.
65
66 However, for freshwater phytoplankton, we found that the uptake of Cd remained unchanged
67 at constant free $Cd^{2+}$ concentrations buffered by the strong ligand NTA, even when cysteine
68 was added and the concentrations of the Cd-cysteine complexes were higher than those of the
69 free $Cd^{2+}$ ('two-ligand' system).[12] More interestingly, in media buffered by a single ligand
70 (referred to as a 'one-ligand' system), Cd uptake in the presence of cysteine alone can be
71 consistently higher, lower, or unchanged in comparison to Cd uptake in the presence of NTA
72 alone; the specific effect of cysteine on Cd uptake depends upon nitrogen nutrition (i.e., the
73 form of nitrogen used as a nutrient for algal growth).[13]
74

75 Several hypotheses have been advanced to explain these 'apparently contrasting' results,
76 including: 1) that the enhanced metal uptake was due to uptake of intact metal-LMW
77 complexes via membrane transporters normally dedicated to transport of the free LMW
78 ligand, such as amino acid transporters; however, tests with unnatural D-amino acids ruled out
79 this explanation;[8, 12] and 2) that the enhancement was due to the formation of a ternary
80 complex with metal transporters (i.e., ligand exchange between the metal-LMW complex and
81 membrane transporters for the metal), but the tests with several LMW thiols in freshwater
82 algae[12] did not support this hypothesis.
83
84 In our recent papers, we have proposed a third explanation, namely that the foregoing results
85 could be explained by metal speciation changes in the boundary layer surrounding the algal
86 cells (i.e., the phycosphere), such as the degradation of metal-LMW complexes induced by
87 certain nitrogen nutrition regimes and extracellular products.[12, 13] The newly liberated free
88 metal ions would be available for algal uptake if they were not immediately complexed by
89 ambient reactive strong ligands (**Graphical abstract**). In the specific case of cysteine and
90 histidine, the most-studied LMW ligands, metal-ligand complexes and amino acids in the
91 microenvironment can be degraded by reaction with extracellular reactive oxygen species (e.g.
92 $H_2O_2$) and/or by extracellular deamination. Such reactions have been reported for
93 phytoplankton cells cultured with organic nitrogen or nitrate as sources of nitrogen[14, 15] and
94 they would liberate free metal ions in the phycosphere.
95
96 Specifically, in EDTA-buffered seawater any free metal ions released from metal-LMW
97 complexes in the phycosphere would not be immediately complexed by EDTA, because EDTA
98 is largely bound to Ca or Mg and metal-ligand exchange between free metal ions and Ca/Mg-
99 EDTA is slow.[16, 17] It follows that at least some of the newly liberated free metal ions would be
100 quickly taken up by cells, leading to an overall enhancement of metal uptake (**Graphical
101 abstract**). In contrast, in solutions with much less Ca and Mg such as in freshwater, any newly
102 liberated free metal ions in the phycosphere would be immediately complexed by excess free
103 or protonated strong ligands (referred to henceforth as 'immediately reacting ligands', which

104 can include Na/K-bound ligands as well), and therefore no enhancement in metal uptake
105 would be observed in freshwater systems (**Graphical abstract**). However, this hypothesis
106 remains untested.
107
108 In the present work, we designed a series of short-term Cd uptake experiments with a
109 euryhaline chlorophyte *Chlamydomonas reinhardtii* to verify the hypothesis that Ca-induced
110 differences in boundary metal-ligand exchange kinetics could explain the different effects of
111 cysteine on metal uptake in freshwater and marine exposures. Specifically, we invoke a
112 'phycosphere effect' and hypothesize that 1) cysteine-enhanced metal uptake would be
113 observable in the presence of few 'immediately reacting strong ligands', achieved by adding
114 high amounts of Ca or removing strong ligands; and 2) this enhancement would disappear
115 when cysteine degradation in the phycosphere is inhibited or when 'immediately reacting
116 strong ligands' are sufficiently available to complex newly liberated free metal ions, a situation
117 achieved by reducing Ca levels. Also, a marine diatom *Thalassiosira weissflogii* was used to
118 test the hypothesis but with fewer experimental designs, since the cysteine effect on metal
119 uptake had previously been investigated in this species.[8-10]
120
121 Synthetic exposure solutions with different levels of Ca were prepared to control the
122 speciation of both metals and ligands. The euryhaline alga *C. reinhardtii* was pre-acclimated to
123 low or high levels of Ca over months before carrying out Cd uptake tests at the corresponding
124 Ca levels. The marine diatom *T. weissflogii* was studied at a known Ca concentration in the
125 present study, which allowed us to calculate speciation of EDTA and Ca and test the influence
126 of 'slow exchange kinetics between Ca-EDTA and free metal ions' on metal uptake. The alga
127 and the diatom were pre-acclimated to cysteine or ammonium as nutrient sources, because
128 local degradation of amino acids can be stimulated by supplying organic nitrogen as the
129 nitrogen nutrient source or be inhibited by supplying ammonium as the nutrient source.[12, 18, 19]
130

131 **Materials and Methods**

132 Model organisms

133 The model freshwater alga *C. reinhardtii* (strain CPCC11) and the marine diatom *T. weissflogii*
134 (CCMP1336) were obtained from the Canadian Phycological Culture Centre at the University of
135 Waterloo, Canada and the National Center for Marine Algae and Microbiota at Bigelow
136 Laboratory for Ocean Sciences, USA, respectively. Both of them are aseptic strains, and
137 potential bacterial contamination was checked regularly by plating onto nutrient agar
138 throughout the study.
139
140 The artificial freshwater medium MHSM was used to grow *C. reinhardtii*,[20] whereas the
141 artificial seawater AQUIL was prepared for *T. weissflogii*.[21] In these culture media, ammonium
142 is the only N source for *C. reinhardtii* (note that strain CPCC11 cannot utilize nitrate since it
143 carries the *nit1* and *nit2* mutations) whereas nitrate is the N source for *T. weissflogii*. The algae
144 were grown in environmental growth chambers (Conviron, CMP3023) with an illumination of
145 80-100 $\mu mol \cdot m^{-2} \cdot s^{-1}$, at 20 °C and with an agitation of ~100-120 rpm. The details of algal
146 culture and medium preparation can be found in the supporting information (SI). All stock
147 solutions were prepared under a laminar flow hood and filtered through 0.2-µm
148 polycarbonate filters (Merck Millipore Ltd.), and laboratory ware was acid-cleaned (24 h in
149 10% $HNO_3$, Fisher Scientific), rinsed with Milli-Q water at least four times, and dried in a
150 laminar flow hood before use. Chemicals of ACS grade or greater purity were purchased from
151 Sigma-Aldrich unless otherwise noted.

152 Modification of Ca and N nutrition

153 In order to perform a Cd uptake test with *C. reinhardtii* at a high level of Ca, the algae had to
154 be gradually acclimated to the high Ca concentration. To do so, the algae were initially grown
155 in MHSM medium (containing 0.068 mM $Ca(NO_3)_2$) and then the concentration of $Ca(NO_3)_2$
156 was increased progressively in the sequence 0.5, 5.0, 5.3, 7.6, 13.8, 20, 25, 30, 35, 40, up to 50
157 mM Ca (this progressive acclimation lasted 2 months). The 50 mM Ca concentration is higher
158 than the typical concentration in seawater (e.g., 11 mM Ca); however, such a high Ca level is
159 required to minimize the 'immediately reacting' ligands in the exposure medium for metal

160 uptake tests. Another advantage of working at 50 mM Ca is that the ionic strength of the high
161 Ca exposure medium is 0.15 M, which corresponds to the ionic strength at which the stability
162 constants for Cd-cysteine complexes were determined[22] and thus minimizes the uncertainty of
163 the speciation calculations. The algae were transferred to a higher Ca level only when
164 substantial growth was detected with an electronic particle counter (MultisizerTM 3 Coulter
165 Counter®; Beckman). Before use for the Cd uptake tests, the cells had been acclimated at 50
166 mM Ca over months (maximum one year) in order to stabilize their physiological status.
167
168 We observed that *C. reinhardtii* cells that had been acclimated to high Ca concentrations
169 tended to settle down at the bottom of the culture flasks, even when the flasks were agitated.
170 After a one-year acclimation, the cell diameter (~ 5.6 μm) was comparable to that of the cells
171 grown at normal Ca concentrations. On the other hand, after the long-term acclimation at 50
172 mM Ca, visible crystals were observed under the microscope (40 ×) and some cells grew on the
173 particle surfaces. The particles were likely formed by precipitation of calcium phosphate;
174 equilibrium calculations with MINEQL+ 4.62 predict the precipitation of hydroxylapatite (a
175 significant loss of dissolved P but not of Ca). However, this should not greatly affect algal
176 growth since the medium was frequently refreshed and the precipitation is relatively slow. The
177 potential influence on the Cd uptake tests of any inadvertent carry-over of crystal particles
178 from the algal culture medium into the exposure medium was also investigated (see below).
179
180 For some of the Cd uptake tests, to favour extracellular cysteine degradation and local free
181 $Cd^{2+}$ liberation, the *C. reinhardtii* cells were supplied with 5 mM L-cysteine as the sole N source
182 in both high-Ca and low-Ca MHSM media. The algae were acclimated to cysteine for 2 to 3
183 days before the uptake experiments since our preliminary tests showed that cysteine cannot
184 support long-term (i.e., weeks to months) growth of this alga under these culture conditions.
185 However, it has been shown that cysteine can support its growth over 5 days.[23] Similarly, the
186 marine diatom was also pre-acclimated in AQUIL medium with 5 mM L-cysteine as the sole N
187 source (i.e., nitrate was removed) for 5 days. The purpose of the cysteine additions was to
188 stimulate extracellular generation of ROS and/or deamination of amino acids, which would

189 result in cysteine degradation in the phycosphere. Recent works[15, 24, 25] show that both *T.*
190 *weissflogii* and *C. reinhardtii* can release ROS into extracellular solution, and that stressed cells
191 produce more ROS than normally cultured ones. Note that the coastal diatom *T. weissflogii*
192 was not cultured under metal limitation conditions since there is no evidence for metal
193 limitation of growth such as Zn in its natural habitats.[26]

194 Exposure media

195 Given that our exposures were for short periods (~ 4 h), we used a simplified version of the
196 algal growth media as the basis for the exposure media (see SI for the complete chemical
197 composition of the exposure media). To simplify the control of the speciation of metals and
198 ligands and avoid precipitation, the phosphate and the trace metal-EDTA solutions were not
199 added to the exposure solution for *C. reinhardtii* (i.e., no possible calcium phosphate
200 precipitation in the high-Ca media), and the major nutrients (N, P and Si), trace metals and
201 vitamins were excluded from the exposure medium for *T. weissflogii*. The pH of the exposure
202 medium for *C. reinhardtii* tests was well buffered at 7.0 with 10 mM 3-(N-
203 morpholino)propanesulfonic acid (MOPS), whereas no pH buffer was added into the relatively
204 stable seawater AQUIL medium for *T. weissflogii*.
205
206 Carrier free radioactive $^{109}Cd$ of very high specific radioactivity (365 Ci $g^{-1}$, Eckert & Ziegler,
207 California) was used to achieve environmentally realistic metal concentrations and to be able
208 to follow Cd uptake at free $Cd^{2+}$ concentrations ranging from around 6 to 64 pM. A stable Cd
209 solution (1 mg $L^{-1}$, in 0.5% $HNO_3$, Optima, Fisher Scientific) was prepared by dilution of a 1 g Cd
210 $L^{-1}$ standard solution (PlasmaCAL, SCP Science) to reach the final concentrations of total
211 dissolved Cd in exposure media (20 nM or 50 nM), and the final ratio of $^{109}Cd$ to total Cd was
212 less than 3.1%. The stock solutions of EDTA and cysteine were freshly prepared no more than
213 24 h prior to the metal uptake tests, and both solutions were filtered through 0.2-µm
214 polycarbonate membrane filters prior to use. Our previous studies had demonstrated that
215 oxidation of cysteine in the bulk medium was negligible under these experimental
216 conditions.[12]
217

218 The $^{109}Cd$ and stable Cd were firstly equilibrated with 5 mL aliquots of exposure medium with
219 or without cysteine for 2.5 to 17 hours at 4 °C in the dark, and then the solutions were mixed
220 with exposure medium pre-equilibrated with EDTA for a further 3.5 to 20 h at room
221 temperature in the dark (‘two-ligand’ system). At high Ca levels, EDTA, as a metal buffer, is a
222 better choice than NTA, because it binds trace metals more strongly than does NTA[21] and also
223 because of the known slow coordination reactions between Ca-EDTA and trace metal ions.[17]
224 On the other hand, exposure media with a single metal buffer ligand (‘one-ligand’ system)
225 were prepared for the cysteine-cultured alga and diatom, to confirm that the pre-acclimation
226 to cysteine could enhance cysteine degradation and local liberation of free metal ions.
227
228 The pH of exposure solutions was checked again just before the uptake tests. The exposure
229 solutions were filtered a second time (0.2 µm) in some tests to check if any precipitate had
230 formed. Aliquots of 1 mL of each exposure solution were collected and placed in glass vials
231 pre-filled with 4 mL of Milli-Q water for radioactivity counting.
232
233 Calculation of metal and ligand speciation
234 Chemical speciation in the exposure medium was calculated with chemical equilibrium
235 software (MINEQL+ 4.62 – Schecher and McAvoy [27]); the default stability constants in the
236 software database were used except in the case of MOPS, for which the values reported by
237 Soares et al. [28] were used. The nominal concentrations of cations, anions, and ligands as well
238 as the determined pH were used for the calculations, and the formation of solid phases was
239 considered.
240
241 Short-term Cd uptake tests
242 The algae were harvested from the growth culture by filtration onto 2-µm polycarbonate
243 filters, and rinsed with a solution similar to the exposure solution but without addition of Cd
244 and ligands (see SI for the chemical composition of the rinse solution). Ten successive rinses
245 were used to remove trace amounts of the extracellular culture solution. The algal cells were
246 then resuspended into 10 mL of the rinse solution, and 0.1 mL of algal suspension was taken

247 to determine cell density with the particle counter. In the cases of the high Ca culture medium
248 where *C. reinhardtii* was associated with crystal particles, the algal cells were counted with a
249 hemocytometer under a light microscope.[29]
250
251 To initiate the uptake, small aliquots of algae were added into flasks with the exposure
252 medium (150 or 200 mL), and the flasks were placed in the growth chamber under the same
253 light, temperature and agitation conditions as for the algal culture; for each experimental
254 condition we used three replicate flasks. Over the following 4 hours, 50 mL of exposure
255 solution from each replicated flasks were filtered onto two superimposed 2-μm filters (the
256 lower filter was used to correct for any passive retention of Cd by the filters) every 0.5 to 1.5
257 hours, and the cells were soaked for 5 min (*T. weissflogii*) or 10 min (*C. reinhardtii*) with 10 mL
258 rinse solution containing EDTA (*C. reinhardtii*) or EDTA + oxalate (*T. weissflogii*) to remove
259 extracellular Cd.[30, 31] The chemical composition of these rinse solutions can be found in the SI.
260 Since ambient high Ca might hinder extracellular metal removal by EDTA (due to the slow
261 metal-ligand exchange kinetics), Ca was replaced by Na in the rinse solution for *C. reinhardtii*
262 acclimated to high-Ca conditions whereas oxalate was added to the rinse solution for *T.*
263 *weissflogii* following an established protocol for rinsing marine diatoms.[31] After another two
264 rinses, the cells associated with the upper filter and the lower filter were separately put into
265 glass vials pre-filled with 5 mL of water. The radioactivity of the collected samples was
266 determined between 16 and 32 keV for 10 min with a gamma counter (Wallac Wizard2, Perkin
267 Elmer). The intracellular Cd amount was calculated based upon the specific radioactivity of
268 $^{109}$Cd in the exposure medium and the difference in $^{109}$Cd amounts between the upper filter
269 and the lower filter.
270
271 <u>Statistical analyses</u>
272 The data were analyzed with statistical software (SPSS 16.0; SigmaPlot 12.5 for Windows).
273 Specifically, the linear regressions for the short-term Cd uptake rates were calculated with
274 SigmaPlot whereas a general linear model in SPSS was employed to compare the uptake rates

275 between treatments for the same algal batch. The significance level was set at $p < 0.05$, unless
276 otherwise noted. The figures were prepared using the ggplot2 package of R software (V 3.4.4).
277

278 **Results**

279 <u>Effect of cysteine on Cd uptake by high-Ca acclimated freshwater algae</u>
280 Cadmium accumulation in the 'two-ligand' system by the freshwater alga *C. reinhardtii* was
281 linear over up to 4 h (**Fig. 1**), indicating that Cd efflux was not significant under the tested
282 conditions. For the high Ca acclimated algae pre-cultured with cysteine as a N source and
283 exposed to around 60 pM $Cd^{2+}$ buffered by 20 µM EDTA and with 50 mM Ca, the Cd uptake
284 rate in the presence of 630 pM Cd-cysteine complexes was 2.2-fold higher than that in the
285 absence of cysteine ('two-ligand' system, $p < 0.01$; **Fig. 1A** & **Table 1**). However, at low Ca (e.g.,
286 0.07 mM) cysteine addition did not enhance Cd uptake by the algae pre-cultured with cysteine
287 ('two-ligand' system, **Fig. 1B** & **Table 1**). Moreover, for the high-Ca acclimated algae that had
288 been grown with ammonium ion as the N source and were exposed to around 60 pM $Cd^{2+}$, the
289 Cd uptake rate in the presence of either 250 or 630 pM Cd-cysteine complexes did not differ
290 from that in the absence of cysteine ('two-ligand' system, $p > 0.05$; **Fig. 1C** & **Table 1**).
291

292 Under the same exposure conditions but in the 'one-ligand' system, the Cd uptake rate in the
293 presence of cysteine alone was 6.0-fold higher than that in the presence of EDTA in these
294 cysteine-cultured algae (**Fig. S1**), indicating the supply of cysteine as the N source had
295 successfully induced (or enhanced) cysteine degradation and free $Cd^{2+}$ liberation.
296

297 During these experiments, we also collected, washed and analyzed the crystals that were
298 present in the algal culture medium and might have inadvertently been included with the algal
299 inoculum. Although the Cd associated with the crystal particles did tend to increase slightly
300 over time (**Fig. 2A**), the amount of Cd associated with the crystals was much less than that
301 associated with the algae (**Fig. 2B**). Note too that the accumulation of Cd by the crystal
302 particles in the presence of cysteine was similar to that in the absence of cysteine ($p > 0.05$;

303 **Fig. 2A**). Overall, the potential interference from the crystal particles on the measurement of
304 algal Cd uptake was deemed negligible.
305
306 Effect of cysteine on Cd uptake by marine algae
307 For the marine diatom *T. weissflogii* that had been pre-cultured with cysteine as a N source,
308 the Cd uptake rate at a bulk concentration of 6 pM $Cd^{2+}$ in the presence of cysteine alone
309 ('one-ligand' system) was 2.9-fold higher than that in the presence of EDTA alone ($p < 0.01$, **Fig.**
310 **S2**). However, in 'two-ligand' system, at the same bulk concentration of 6 pM $Cd^{2+}$ in the
311 presence of 550 pM Cd-cysteine complexes, the Cd uptake rate by these cysteine-cultured
312 diatoms did not significantly differ from that in the absence of cysteine ($p > 0.05$; **Fig. 3** and
313 **Table 1**).
314

315 **Discussion**
316 The question we addressed in this work is why, in a 'two-ligand' exposure system, LMW
317 ligands such as cysteine can enhance uptake of trace metals by marine phytoplankton but not
318 by freshwater species. We focus here on the untested hypothesis, i.e., Ca-induced differing
319 metal-ligand exchange kinetics within the phycosphere in marine and freshwater
320 environments, because neither 'uptake of metal-cysteine complexes' nor 'formation of ternary
321 complexes with metal transporters' can consistently explain these results.
322
323 It should be noted that the concentrations of the free metal ions, including $Cd^{2+}$ and other
324 major cations in the bulk exposure solution, were always kept the same and constant for
325 different cysteine treatments in a given test. The exposure media were chemically stable
326 within this experimental period and the same algal batch was used for a given test; in other
327 words, any changes in metal uptake rates cannot plausibly be attributed to chemical changes
328 in the bulk solution or to changes in algal physiology.
329

330 Metal-ligand exchange kinetics in the phycosphere of freshwater algae

331 Our previous study suggested that the disappearance of enhanced Cd uptake in the 'two-
332 ligand' (strong ligand + weak ligand cysteine) freshwater exposure solutions was due to an
333 intrinsic fast coordination reaction between the excess 'immediately reacting' strong ligand
334 and free $Cd^{2+}$; any local increase of free $Cd^{2+}$ newly liberated from cysteine would be rapidly
335 suppressed in the presence of excess 'immediately reacting' strong ligand.[12]

336

337 In theory, by increasing the Ca concentration in the 'two-ligand' medium to slow down the
338 metal-strong ligand exchange kinetics, an enhancement in Cd uptake by freshwater algae
339 should re-appear in the presence of cysteine. Given the known slow exchange kinetics
340 between Ca-EDTA and free metal ions,[17] we used EDTA as the metal buffer to test this
341 hypothesis experimentally. As is the case in seawater, the coordination reaction between $Cd^{2+}$
342 and Ca-EDTA would be slow in this high-Ca 'freshwater' medium, and any increase in free $Cd^{2+}$
343 in the phycosphere would potentially increase its uptake by the algal cells. To test this idea,
344 the dominant form of EDTA in the exposure solution (and in the phycosphere) must be Ca-
345 EDTA (or Mg-EDTA), and more importantly any trace of 'immediately reacting' EDTA species
346 should be insufficient to sequester newly liberated free $Cd^{2+}$ before it can be taken up by the
347 cells or diffuse toward the bulk solution.

348

349 In the present study, we prepared this high-Ca medium and carried out Cd uptake tests with *C.*
350 *reinhardtii* that had been acclimated to the high-Ca freshwater. As predicted, enhanced Cd
351 uptake was observed in the presence of cysteine at 50 mM Ca (**Fig. 1A**). In this 'two-ligand'
352 medium, nearly 100% of the EDTA was complexed by Ca, and the concentration of
353 'immediately reacting' EDTA (i.e., 51 pM) was much lower than the concentration of total Cd-
354 cysteine complexes (i.e., 630 pM) (**Table 1**). In experiments with *C. reinhardtii* that had been
355 acclimated to high Ca and pre-exposed to cysteine, we also observed enhanced Cd uptake in
356 the high-Ca exposure medium in the presence of cysteine alone (i.e., when EDTA was removed
357 but free $Cd^{2+}$ was kept constant), in comparison to the uptake observed in the presence of
358 EDTA alone (i.e., 'one-ligand' system, **Fig. S1**). This result is consistent with our previous

359 studies in low-Ca exposure media with *C. reinhardtii* pre-exposed to cysteine[12, 13] and it
360 indicates that local liberation of free $Cd^{2+}$ is not influenced by the high-Ca acclimation but is
361 closely linked to nitrogen nutrition.
362
363 The absence of enhanced Cd uptake in the high-Ca exposures by *C. reinhardtii* that had been
364 grown with ammonium ion was also expected. Similar results were obtained in uptake
365 experiments with *C. reinhardtii* that had been cultivated with $NH_4^+$ as the nitrogen source but
366 exposed to $Cd^{2+}$ in a low-Ca environment,[12] and with cysteine-acclimated *C. reinhardtii* in the
367 presence of ROS scavengers.[13] It is known that the presence of the ammonium ion inhibits
368 extracellular degradation of amino acids[19, 32] and associated ROS production,[18] and thus there
369 would be minimal local cysteine oxidation and no change in the free $Cd^{2+}$ concentration in the
370 phycosphere of the ammonium-acclimated cells.
371
372 Overall, we show that supplying an organic nitrogen source such as cysteine and increasing the
373 ambient Ca concentration (or removing strong ligands) could enhance the bioavailability of Cd-
374 cysteine, whereas either supplying ammonium or decreasing ambient Ca could eliminate the
375 effect of cysteine on Cd bioavailability. These results suggest that an enhanced metal uptake in
376 the presence of LMW ligands would be observable when there is a significant local enrichment
377 of free metal ions in the phycosphere and when strong ligands cannot immediately complex
378 these free metal ions.
379
380 <u>Metal-ligand exchange kinetics in the phycosphere of marine algae</u>
381 In previous studies using field-collected seawater, significantly enhanced uptake of trace
382 metals in the presence of LMW ligands including cysteine has been observed with marine
383 phytoplankton, especially those that have been cultured under metal-limiting conditions.[8, 9, 11]
384
385 Theoretically, even in seawater, the phenomenon of enhanced metal uptake would disappear
386 if any free metal ions newly liberated from metal-LMW complexes were immediately
387 sequestered by a substantial local concentration of ‘immediately reacting’ strong ligands, or

388 when the local enrichment of free metal ions was insignificant. To test this idea, not only
389 metal speciation but also the speciation of ligands has to be well controlled, notably their
390 complexation with Ca and Mg. In the present study, the artificial seawater medium AQUIL was
391 used for metal uptake experiments with the marine diatom. In this medium, the concentration
392 of the 'immediately reacting' EDTA (i.e., 7.5 nM) was much higher than the total concentration
393 of Cd-cysteine complexes (i.e., 0.55 nM). In other words, the 'immediately reacting' EDTA
394 would likely be sufficient to sequester any free $Cd^{2+}$ released from Cd-cysteine complexes and
395 thus inhibit any enhanced metal uptake.
396
397 The present results are consistent with this prediction; we found that the addition of cysteine
398 to the AQUIL-based exposure medium did not enhance Cd uptake by cysteine-acclimated *T.*
399 *weissflogii* at all, even when the total concentration of Cd-cysteine complexes was 92-fold
400 higher than the free $Cd^{2+}$ concentration. The disappearance of the cysteine effect was not due
401 to insignificant cysteine degradation. In an experiment at the same free $Cd^{2+}$ concentration,
402 but where EDTA was removed from the exposure medium and cysteine remained, the Cd
403 uptake rate was higher than in the EDTA-buffered medium ('one-ligand' system, **Fig. S2**),
404 indicating that cysteine pre-acclimation had significantly induced cysteine degradation and
405 $Cd^{2+}$ liberation. The lack of enhanced metal uptake in the presence of cysteine in 'two-ligand'
406 systems has also been observed in some earlier tests, particularly with metal-replete marine
407 phytoplankton.[8, 10] The failure to detect cysteine enhanced-metal uptake in these earlier cases
408 might result from an insignificant increase of the free metal ion concentration in the
409 phycosphere under the experimental conditions.
410
411 In addition to the nitrogen source, the state of the algal cells with respect to other trace
412 metals such as Zn and Cu might also play a role in the algal utilization of metals bound to LMW
413 metabolites. The enhancement effect of LMW ligands on metal uptake was shown to be more
414 dramatic for metal-limited cells than for metal-replete cells.[8, 10, 11] We speculate that
415 phycosphere degradation of LMW ligands might be more significant in metal-limited cells than
416 in the metal-replete cells, and thus that there would be more significant enhancement of the

417 local concentration of free metal ions in the former case. However, metal limitation does not
418 appear to be a precondition for the enhanced metal uptake by weak ligands in either marine
419 or freshwater phytoplankton, since enhanced metal uptake was reported in Zn-replete *T.*
420 *weissflogii* ('two-ligand' system),[33] in metal-replete *T. weissflogii* ( 'one-ligand' system, **Fig. S2**),
421 and in metal-replete *Pseudokirchneriella subcapitata* and *Anabaena flos-aquae* ('one-ligand'
422 system).[12]
423

424 Environmental implications

425 The nature of naturally occurring organic ligands in real-world waters is poorly defined, and
426 chemically they have been roughly classified into two groups (i.e., strong ligands such as
427 siderophores and weak ligands such as LMW ligands, humic and fulvic acids).[34, 35] Except for
428 certain groups of prokaryotes,[36] there is no direct evidence that phytoplankton cells can
429 directly assimilate metal-ligand complexes; free metal ions are directly accessible by all of the
430 tested organisms.[5]
431

432 Historically, it has been assumed that for many cationic metals, the presence of organic
433 ligands in natural waters leads to a decrease in the proportion of the dissolved metal that is
434 present as the free metal ion. Normally this complexation would be expected to result in a
435 decrease in metal bioavailability; indeed, addition of cysteine in 'one-ligand' systems has been
436 shown to significantly reduce Cd uptake[12] and Ag toxicity,[37] in comparison to solutions with
437 the same concentration of total dissolved metals but in the absence of cysteine. However, as
438 has been shown in the present study and in other work on LMW ligands,[9, 38] degradation of
439 weak ligands in the phycosphere may significantly weaken metal complexation under certain
440 situations and thus enhance metal uptake; in these cases, metals associated with weak ligands
441 can be an important pool for uptake. Unlike the situation with the coastal diatom *T.*
442 *weissflogii*, where Cd can substitute for Zn in carbonic anhydrase[39] under metal-limited
443 conditions, there is no evidence that enhanced uptake of Cd by the freshwater *C. reinhardtii*
444 could be beneficial to this alga. In other words, phycosphere chemistry could potentially
445 increase metal toxicity or improve nutrition of those essential trace metals such as Fe, Zn and

446 Cu; neither of these possible effects can be predicted solely on the basis of the chemistry of
447 the ambient bulk water.
448
449 One of the implications of our work is that the use of synthetic strong ligands such as EDTA or
450 NTA as metal buffers may have unintended consequences when they are used in either
451 freshwater or marine systems. For instance, the enhancement of Cd uptake in the presence of
452 cysteine could not be detected with the freshwater algae unless these 'strong ligands' were
453 removed (i.e., 'one-ligand' system) or were all tied up with excess calcium (i.e., 'two-ligand'
454 system). Such synthetic ligands will also be absent in most natural waters and in such cases, if
455 the binding sites of the natural ligands for Ca and trace metals are different, as might be
456 expected in a polymeric or supramolecular ligand, or if the affinity of the ligand for Ca is weak
457 but strong for trace metals, as has been shown for humic acids,[40] high ambient Ca
458 concentrations would not hinder the coordination reactions between the ligand and trace
459 metals and thus would not facilitate the utilization of weak-ligand bound metal by cells. It
460 remains speculative whether or not the binding sites of naturally occurring organic ligands for
461 Ca differ from those for trace metals; for instance, they might be different for allochthonous
462 organic matter (e.g. in freshwater) but less so for autochthonous organic matter (e.g., in
463 seawater).
464
465 Much is known about the influence of abiotic factors (i.e., bulk water chemistry) and biotic
466 processes (i.e., cell membrane biochemistry and regulation of metal transporters) on trace
467 metal uptake. The present results suggest that reactions at the interface between these two
468 realms, i.e., in the boundary layer separating the algal cell from its abiotic environment, may
469 also play a role in determining metal bioavailability to phytoplankton.
470

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476 supported a Newton International Fellowship (Royal Society of the United Kingdom) during
477 the preparation of the manuscript.

478

479 **Supporting Information**

480 Preparation and chemical composition of culture and exposure media, and rinse solutions.
481 Figures showing short-term Cd uptake rates in 'one-ligand' exposure systems by *C. reinhardtii*
482 and *T. weissflogii.*

483

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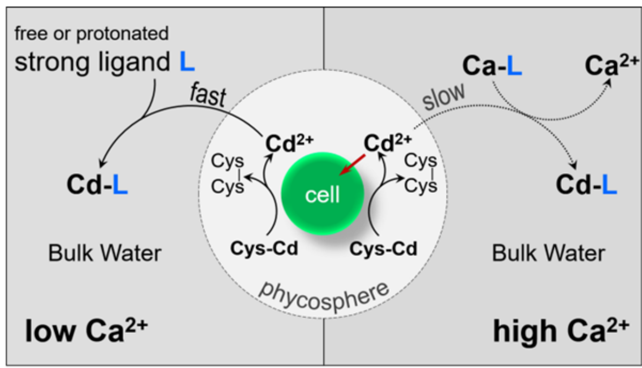
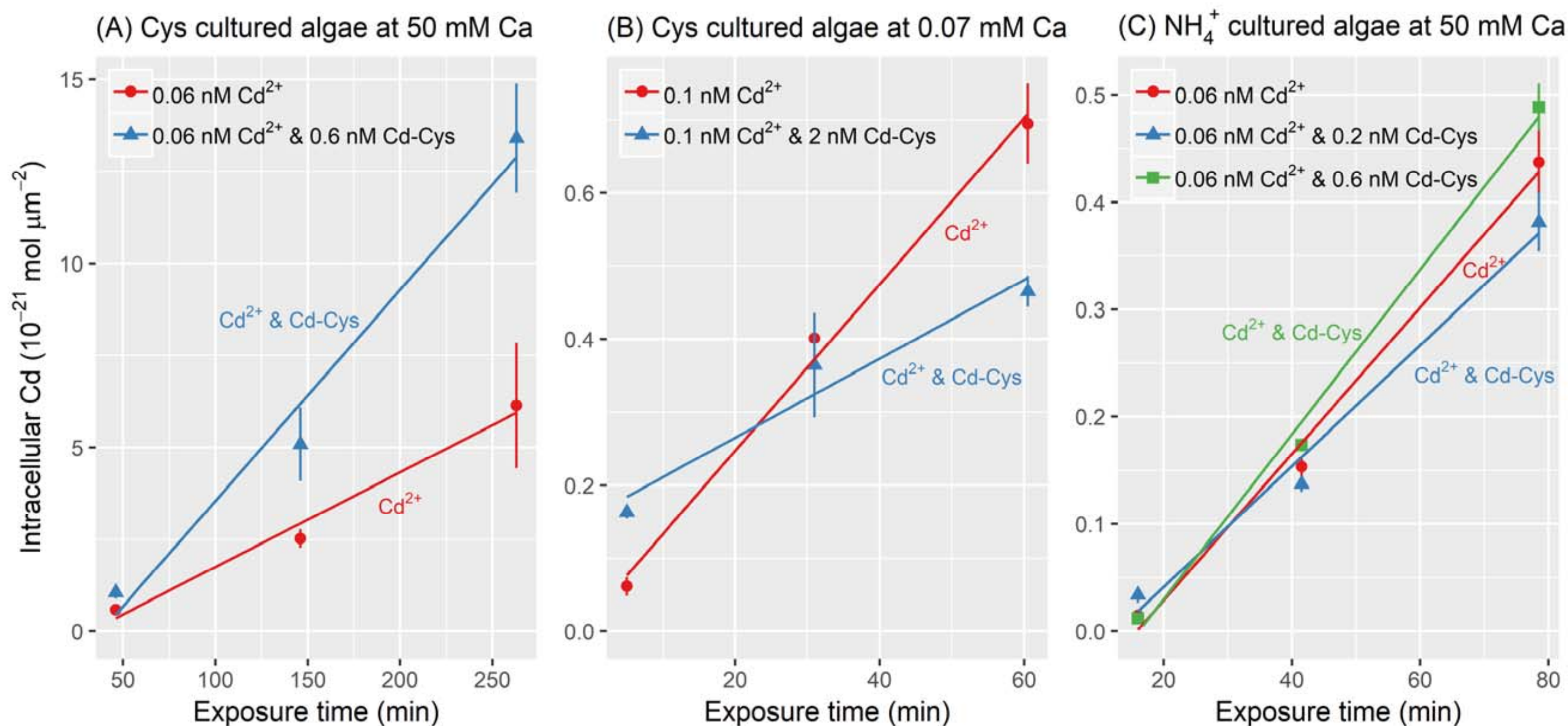



579 **Graphical abstract (TOC)**

580

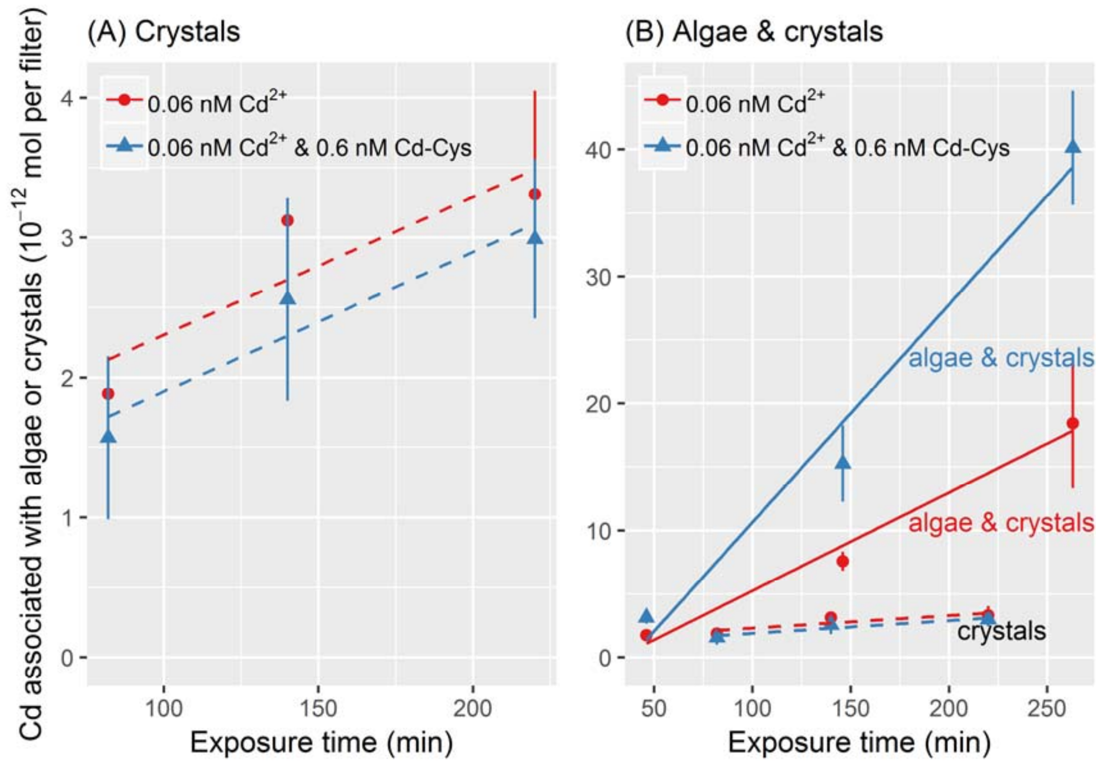



581

582 Figure 1. Short-term Cd uptake (N = 3, mean ± SD) in a 'two-ligand' system (EDTA & cysteine) by the freshwater alga *Chlamydomonas*
583 *reinhardtii* pre-cultured with different nitrogen sources (to modify local $Cd^{2+}$ liberation via cysteine degradation) in media
584 with different levels of Ca (to change metal-ligand exchange kinetics between $Cd^{2+}$ and strong ligands). (**A**) A significantly
585 higher Cd uptake rate was observed in the presence of cysteine (blue triangles) than that in the absence of cysteine (red
586 dots) at 0.06 nM bulk [$Cd^{2+}$] buffered by 20 µM EDTA in the presence of 50 mM Ca (to slow metal-ligand exchange kinetics
587 between $Cd^{2+}$ and EDTA, compared to a low Ca system). The algae were acclimated to the high [Ca] over months and were
588 pre-cultured (3 d) with cysteine as a N source (to enhance cysteine degradation). (**B**) No significant enhancement in Cd
589 uptake rate in the presence of cysteine was observed at 0.1 nM bulk $Cd^{2+}$ buffered by 10 µM NTA in the presence of 0.07
590 mM Ca (to increase metal-ligand exchange kinetics between $Cd^{2+}$ and NTA, compared to a high Ca system); the uptake rate
591 in the presence of cysteine was slightly lower than in the absence of cysteine. The algae were pre-cultured (2 d) with
592 cysteine. (**C**) No significant enhancement in Cd uptake rate in the presence of cysteine was observed at 0.06 nM bulk [$Cd^{2+}$]
593 buffered by 20 µM EDTA in the presence of 50 mM Ca. The algae were acclimated to the high [Ca] (over months) and were
594 pre-cultured with ammonium ion (to inhibit cysteine degradation). Note that panel (B) comes from Liu, Fortin and

595 Campbell [12].

596




597

598 Figure 2. (**A**) The control test shows that cysteine addition did not affect the amount of Cd associated with crystal particles, which
599 were collected from the 50 mM Ca culture medium and washed in the same way as the cells. (**B**) The amount of Cd
600 associated with the crystal particles was much less than that in the presence of *C. reinhardtii* cells. N = 3, mean ± SD.

601

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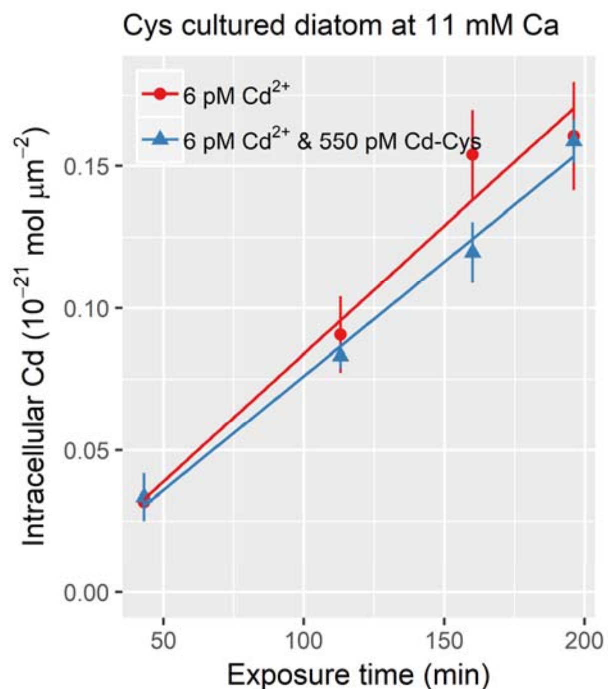


603
604 Figure 3. Short-term Cd uptake (N = 3, mean ± SD) in a 'two-ligand' system (EDTA & cysteine) by the marine diatom *Thalassiosira*
605 *weissflogii*. The diatom was pre-cultured (5 d) with cysteine (to enhance Cd-cysteine degradation) in artificial seawater
606 AQUIL medium (containing 11 mM Ca). The Cd uptake rate in the presence of cysteine (blue triangles) was not
607 significantly different from that in the absence of cysteine (red dots) at 6 pM bulk $Cd^{2+}$ buffered by 100 µM EDTA. The
608 concentration of 'immediately reacting' (i.e., the species that can immediately sequester free $Cd^{2+}$) was 7.5 nM, which
609 was much higher than the total concentration of Cd-cysteine complexes (0.55 nM, see Table 1 for details).
610

Table 1. Summary of the chemical composition of exposure media and algal Cd uptake results.

| | Fig. 1(A) | Fig. 1(A) | | Fig. 1(B) | Fig. 1(B) | | Fig. 1(C) | Fig. 1(C) | Fig. 1(C) | Fig. 3 | Fig.3 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Uptake test*** | | | | | | | | | | | |
| Alga | *C. reinhardtii* | | | *C. reinhardtii* | | | *C. reinhardtii* | | | *T. weissflogii* | |
| Ca level | $5.0 \times 10^{-2}$ | $5.0 \times 10^{-2}$ | | $6.8 \times 10^{-5}$ | $6.8 \times 10^{-5}$ | | $5.0 \times 10^{-2}$ | $5.0 \times 10^{-2}$ | $5.0 \times 10^{-2}$ | $1.1 \times 10^{-2}$ | $1.1 \times 10^{-2}$ |
| N source | cysteine | cysteine | | cysteine | cysteine | | $NH_4^+$ | $NH_4^+$ | $NH_4^+$ | cysteine | cysteine |
| Cd uptake | $2.6 \times 10^{-23}$ | $5.7 \times 10^{-23}$ | | $1.1 \times 10^{-23}$ | $5.4 \times 10^{-24}$ | | $6.8 \times 10^{-24}$ | $5.6 \times 10^{-24}$ | $7.7 \times 10^{-24}$ | $9.0 \times 10^{-25}$ | $8.0 \times 10^{-25}$ |
| Fold change | | 2.2 | | | 0.5 | | | 0.8 | 1.1 | | 0.9 |
| Sig. | | <0.01 | | | <0.01 | | | >0.05 | >0.05 | | >0.05 |
| ***Exposure medium*** | | | | | | | | | | | |
| $Cd^{2+}$ | $6.4 \times 10^{-11}$ | $6.2 \times 10^{-11}$ | | $1.1 \times 10^{-10}$ | $1.0 \times 10^{-10}$ | | $6.4 \times 10^{-11}$ | $6.3 \times 10^{-11}$ | $6.2 \times 10^{-11}$ | $6.1 \times 10^{-12}$ | $6.0 \times 10^{-12}$ |
| Cd-Cys | 0.0 | $6.3 \times 10^{-10}$ | | 0.0 | $2.1 \times 10^{-9}$ | | 0.0 | $2.5 \times 10^{-10}$ | $6.3 \times 10^{-10}$ | 0.0 | $5.5 \times 10^{-10}$ |
| Total Cd | $2.0 \times 10^{-8}$ | $2.0 \times 10^{-8}$ | | $2.0 \times 10^{-8}$ | $2.0 \times 10^{-8}$ | | $2.0 \times 10^{-8}$ | $2.0 \times 10^{-8}$ | $2.0 \times 10^{-8}$ | $5.0 \times 10^{-8}$ | $5.0 \times 10^{-8}$ |
| Total EDTA | $2.0 \times 10^{-5}$ | $2.0 \times 10^{-5}$ | Total NTA | $1.0 \times 10^{-5}$ | $1.0 \times 10^{-5}$ | Total EDTA | $2.0 \times 10^{-5}$ | $2.0 \times 10^{-5}$ | $2.0 \times 10^{-5}$ | $1.0 \times 10^{-4}$ | $1.0 \times 10^{-4}$ |
| Ca-EDTA | $2.0 \times 10^{-5}$ | $2.0 \times 10^{-5}$ | Ca/Mg-NTA | $3.9 \times 10^{-6}$ | $3.9 \times 10^{-6}$ | Ca-EDTA | $2.0 \times 10^{-5}$ | $2.0 \times 10^{-5}$ | $2.0 \times 10^{-5}$ | $^{\&}1.0 \times 10^{-4}$ | $^{\&}1.0 \times 10^{-4}$ |
| *EDTA | $5.1 \times 10^{-11}$ | $5.1 \times 10^{-11}$ | *NTA | $6.1 \times 10^{-6}$ | $6.1 \times 10^{-6}$ | *EDTA | $5.1 \times 10^{-11}$ | $5.1 \times 10^{-11}$ | $5.1 \times 10^{-11}$ | $7.5 \times 10^{-9}$ | $7.5 \times 10^{-9}$ |
| Ionic strength | $1.5 \times 10^{-1}$ | $1.5 \times 10^{-1}$ | | $9.2 \times 10^{-3}$ | $9.2 \times 10^{-3}$ | | $1.5 \times 10^{-1}$ | $1.5 \times 10^{-1}$ | $1.5 \times 10^{-1}$ | $6.2 \times 10^{-1}$ | $6.2 \times 10^{-1}$ |
| Solution pH | 7.0 | 7.0 | | 7.0 | 7.0 | | 7.0 | 7.0 | 7.0 | 8.0 | 8.0 |
| pH buffer | MOPS | MOPS | | MOPS | MOPS | | MOPS | MOPS | MOPS | bicarbonate | bicarbonate |
| Other ions | SMHSM | SMHSM | | SMHSM | SMHSM | | SMHSM | SMHSM | SMHSM | AQUIL# | AQUIL# |

Note

Concentrations are expressed in mol $L^{-1}$ for all of the chemical species and the ionic strength.

Cd-Cys includes all Cd-cysteine complexes.

Ca-EDTA (and Mg-EDTA) cannot sequester free metal ions immediately due to their slow exchange kinetics. &This total concentration includes Mg-EDTA.

*EDTA refers as the ‘immediately reacting’ ligands, representing the total concentration of Na-EDTA, K-EDTA, protonated and free EDTA, and they are expected to be able to immediately sequester free $Cd^{2+}$ ions. Total concentrations of Na-EDTA and K-EDTA were similar to the protonated EDTA in the SMHSM medium while Na-EDTA dominated the pool of *EDTA in the AQUIL#.

Similarly, *NTA includes Na-NTA, K-NTA, protonated and free NTA.

The pH of seawater AQUIL media is buffered by the 2 mM bicarbonate and to a lesser extent by borate.

SMHSM is a simplified artificial freshwater medium, while AQUIL# is a simplified version of AQUIL seawater medium. See supporting information for detailed chemical composition.

MOPS, 3-(N-morpholino)propanesulfonic acid.
Cd uptake rates are expressed in units of mol $\mu m^{-2}$ $min^{-1}$.
Fold change, a comparison of Cd uptake rate by the same algal batch between the test in the presence of cysteine and that in the absence of cysteine.

611

Supporting information

Why does cysteine enhance metal uptake by phytoplankton in seawater but not in fresh water?

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4.2 Rinse solutions for the marine diatom *T. weissflogii* .......... S11

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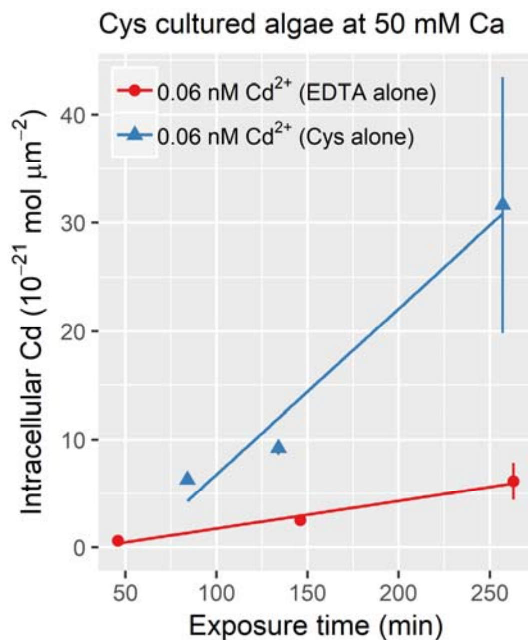


Figure S1. In the ''one-ligand'' exposure system, Cd uptake (N = 3, mean ± SD) in the presence of cysteine alone (blue triangles) was higher than that in the presence of EDTA alone (red dots) at 0.06 nM bulk $Cd^{2+}$ in the 50 mM Ca freshwater MHSM medium. The euryhaline alga *Chlamydomonas reinhardtii* was acclimated to the high Ca concentration (over months) and was pre-cultured with cysteine as the N source (to enhance cysteine degradation).

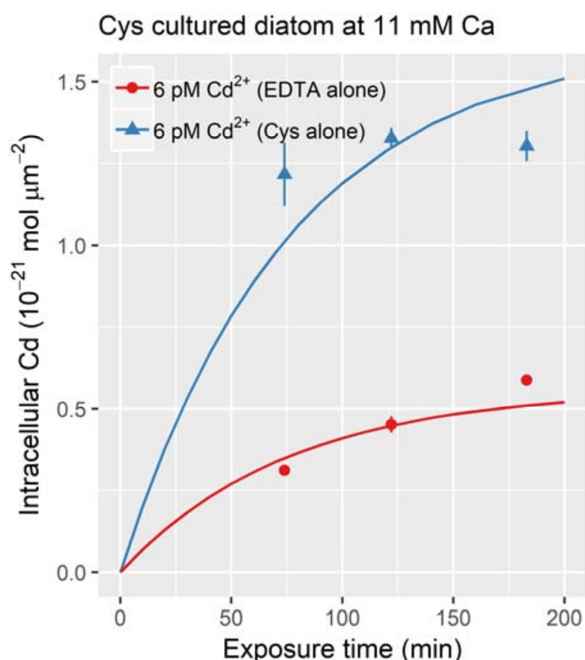


Figure S2. In the ''one-ligand'' exposure system, Cd uptake (N = 3, mean ± SD) in the presence of cysteine alone (blue triangles) was higher than that in the presence of EDTA alone (red dots) at 6 pM bulk $Cd^{2+}$. The marine diatom *Thalassiosira weissflogii* was pre-cultured (5 d) with cysteine in artificial seawater AQUIL medium (containing 11 mM Ca). In the exposure media, total Cd = 50 nM buffered by either 77 µM cysteine or 100 µM EDTA, and pH = 8.0. Note that the rate of increase of intracellular Cd appeared to slow down with the time of exposure, especially in the Cys treatment, indicating a possible efflux of Cd. The same efflux rate constant was assumed for both treatments in fitting the Cd uptake data. The Cd uptake rates were estimated to be $(0.76 \pm 0.10) \times 10^{-23}$ mol $\mu m^{-2}$ $min^{-1}$ and $(2.23 \pm 0.33) \times 10^{-23}$ mol $\mu m^{-2}$ $min^{-1}$ for the EDTA and Cys treatments, respectively. The efflux rate constant was estimated to be $0.0141 \pm 0.0031$ $min^{-1}$.

## 2 Culture media for the freshwater and marine algae

### 2.1 The culture medium for the freshwater alga *Chlamydomonas reinhardtii*

**Table S1. Chemical composition of the high Ca-MHSM culture medium for the green alga *C. reinhardtii* CPCC11 grown under high Ca condition.**

| Stock name | Chemical | Stock solution ($g \cdot L^{-1}$) | Quantity used for 1 L culture medium | Conc. in final medium ($mol \cdot L^{-1}$) |
|---|---|---|---|---|
| Calcium | $Ca(NO_3)_2 \cdot 4H_2O$ | -- | 11.81 g | $5.00 \times 10^{-2}$ |
| Ammonium #2 | $NH_4NO_3$ | 15.0 | 5 mL | $9.37 \times 10^{-4}$ |
| | $MgSO_4 \cdot 7H_2O$ | 4.00 | | $8.12 \times 10^{-5}$ |
| | $Ca(NO_3)_2 \cdot 4H_2O$ | 3.21 | | $6.80 \times 10^{-5}$ |
| Phosphate #2 | $KH_2PO_4$ | 29.6 | 0.25 mL | $5.44 \times 10^{-5}$ |
| | $K_2HPO_4$ | 57.6 | | $8.27 \times 10^{-5}$ |
| $KNO_3$ | $KNO_3$ | 101 | 4 mL | $4.00 \times 10^{-3}$ |
| NaOH | NaOH | 40.0 | 5 mL | $5.00 \times 10^{-3}$ |
| pH buffer | MOPS* | 20.9 | 100 mL | $1.00 \times 10^{-2}$ |
| Trace elements | Trace elements-EDTA* | See the table below | 1 mL | |

* MOPS = 3-(N-morpholino)propanesulfonic acid; EDTA = ethylenediaminetetraacetic acid.

[#] Note, this algal strain cannot utilize nitrate.

| **Table S1 (continued). Trace elements stock solution without any N sources** | | | |
|---|---|---|---|
| Component | Chemical | Stock solution | Conc. in final medium ($mol \cdot L^{-1}$) |
| EDTA | $Na_2EDTA \cdot 2H_2O$ | $0.302\ g \cdot L^{-1}$ | $8.06 \times 10^{-7}$ |
| Fe | $FeCl_3 \cdot 6H_2O$ | $0.160\ g \cdot L^{-1}$ | $5.92 \times 10^{-7}$ |
| B | $H_3BO_3$ | $0.186\ g \cdot L^{-1}$ | $3.01 \times 10^{-6}$ |
| Mn | $MnCl_2.4H_2O$ | $0.415\ g \cdot L^{-1}$ | $2.10 \times 10^{-6}$ |
| Zn | $ZnCl_2$ | $3.31\ g \cdot L^{-1}$ (1000 ×) | $2.43 \times 10^{-8}$ |
| Co | $CoCl_2 \cdot 6H_2O$ | $2.59\ g \cdot L^{-1}$ (1000 ×) | $1.09 \times 10^{-8}$ |
| Mo | $Na_2MoO_4 \cdot 2H_2O$ | $7.26\ g \cdot L^{-1}$ (1000 ×) | $3.00 \times 10^{-8}$ |
| Cu | $CuSO_4 \cdot 5H_2O$ | $0.0176\ g \cdot L^{-1}$ (1000 ×) | $7.04 \times 10^{-11}$ |

To prepare for the ''trace elements-EDTA'' stock solution: first, prepare individual stock solutions of Zn, Co, Mo and Cu (1000 ×); second, into 900 mL Milli-Q water, add $Na_2EDTA \cdot 2H_2O$ and wait until completely dissolved. Slowly add Fe, then B and Mn, and finally 1 mL of the stock solutions (1000 ×) of Zn, Co, Mo and Cu. Bring to 1 L with Milli-Q water and store the solution at 4 °C.

To make one litre of the high Ca-MHSM culture medium: add the seven stock solutions (i.e., Calcium, Ammonium #2, Phosphate #2, $KNO_3$, NaOH, pH buffer and Trace elements) into Milli-Q water, bringing the final volume to 1 L (adjust pH to 7.0 with 1 M HCl/NaOH). Autoclave at 121 °C for 15 min (to ensure no contamination by other living microbes such as bacteria), cool down under a laminar flow hood, and store the solution at 4 °C.

For the low Ca culture medium, the 11.81 g $Ca(NO_3)_2 \cdot 4H_2O$ was not added and the nominal concentration of Ca in the MHSM medium was 0.068 mM.

When L-cysteine is used as the sole N source (i.e., **cysteine-high Ca-MHSM** culture medium), omit the $NH_4NO_3$, the $Ca(NO_3)_2 \cdot 4H_2O$ is replaced by 5 mL 2 $g \cdot L^{-1}$ $CaCl_2 \cdot 4H_2O$ per litre of medium, and add 50 mL 0.2 µm-filtered 0.1 M L-cysteine per litre autoclaved medium (i.e., 5 mM L-cysteine in the final medium).

## 2.2 The culture medium for the marine diatom *Thalassiosira weissflogii*

The artificial seawater AQUIL medium was prepared for *T. weissflogii* as described by Sunda et al. (2005). Note, since we did not aim to grow the diatoms under trace metal limitation conditions, the stock solutions were not passed through a Chelex column to remove trace amounts of trace metals. However, the solutions were prepared and filtered with 0.2 µm membrane filters under a laminar flow hood and all bottles/laboratory ware were acid-cleaned before use. The medium was sterilized in a microwave oven.

For the **cysteine-AQUIL** medium, a 0.2 µm filtered cysteine stock solution (20 mL) was mixed with the sterilized AQUIL medium (980 mL) without addition of $NaNO_3$, and the final concentration of L-cysteine was 5 mM. The freshly prepared cysteine-AQUIL medium was used to acclimate the diatoms.

## 3 Exposure media for short-term Cd uptake tests

**Table S2. Chemical composition of the exposure medium for high Ca pre-acclimated *C. reinhardtii* CPCC11 pre-cultured with cysteine as a N source (i.e., Figures 1A & S1). Total concentrations of the chemical components are shown in mol·L$^{-1}$.**

| Treatment | EDTA+Cd | EDTA+Cys+Cd | Cys+Cd |
|---|---|---|---|
| pH | 7.0 | 7.0 | 7.0 |
| $Cl^-$ | $4.14 \times 10^{-3}$ | $4.14 \times 10^{-3}$ | $4.14 \times 10^{-3}$ |
| $K^+$ | $4.00 \times 10^{-3}$ | $4.00 \times 10^{-3}$ | $4.00 \times 10^{-3}$ |
| $NH_4^+$ | 0.00 | 0.00 | 0.00 |
| $NO_3^-$ | $1.00 \times 10^{-1}$ | $1.00 \times 10^{-1}$ | $1.00 \times 10^{-1}$ |
| $SO_4^{2-}$ | $8.12 \times 10^{-5}$ | $8.12 \times 10^{-5}$ | $8.12 \times 10^{-5}$ |
| $Mg^{2+}$ | $8.12 \times 10^{-5}$ | $8.12 \times 10^{-5}$ | $8.12 \times 10^{-5}$ |
| $Ca^{2+}$ | $5.01 \times 10^{-2}$ | $5.01 \times 10^{-2}$ | $5.01 \times 10^{-2}$ |
| $Na^+$ | $5.00 \times 10^{-3}$ | $5.00 \times 10^{-3}$ | $5.00 \times 10^{-3}$ |
| MOPS | $1.00 \times 10^{-2}$ | $1.00 \times 10^{-2}$ | $1.00 \times 10^{-2}$ |
| total Cd | $2.00 \times 10^{-8}$ | $2.00 \times 10^{-8}$ | $2.00 \times 10^{-8}$ |
| EDTA | $2.00 \times 10^{-5}$ | $2.00 \times 10^{-5}$ | 0.00 |
| Cysteine | 0.00 | $2.00 \times 10^{-5}$ | $1.55 \times 10^{-4}$ |

The ionic strength of the high Ca exposure medium was 0.15 mol·L$^{-1}$, at which the stability constants for Cd-cysteine complexes were determined (Smith et al. 2004). The solutions were open to atmosphere. The algal strain cannot utilize nitrate although nitrate is present in the exposure medium.

**Table S3. Chemical composition of the exposure medium for high Ca pre-acclimated *C. reinhardtii* pre-cultured with ammonium (i.e., Figure 1C). Total concentrations of the chemical components are shown in mol·L$^{-1}$.**

| Treatment | EDTA+Cd | EDTA+Cd +10 µM Cys | EDTA+Cd +20 µM Cys |
|---|---|---|---|
| pH | 7.0 | 7.0 | 7.0 |
| $Cl^-$ | 0.00 | 0.00 | 0.00 |
| $K^+$ | $4.00 \times 10^{-3}$ | $4.00 \times 10^{-3}$ | $4.00 \times 10^{-3}$ |
| $NH_4^+$ | $9.37 \times 10^{-4}$ | $9.37 \times 10^{-4}$ | $9.37 \times 10^{-4}$ |
| $NO_3^-$ | $1.05 \times 10^{-1}$ | $1.05 \times 10^{-1}$ | $1.05 \times 10^{-1}$ |
| $SO_4^{2-}$ | $8.12 \times 10^{-5}$ | $8.12 \times 10^{-5}$ | $8.12 \times 10^{-5}$ |
| $Mg^{2+}$ | $8.12 \times 10^{-5}$ | $8.12 \times 10^{-5}$ | $8.12 \times 10^{-5}$ |
| $Ca^{2+}$ | $5.01 \times 10^{-2}$ | $5.01 \times 10^{-2}$ | $5.01 \times 10^{-2}$ |
| $Na^+$ | $5.00 \times 10^{-3}$ | $5.00 \times 10^{-3}$ | $5.00 \times 10^{-3}$ |
| MOPS | $1.00 \times 10^{-2}$ | $1.00 \times 10^{-2}$ | $1.00 \times 10^{-2}$ |
| total Cd | $2.00 \times 10^{-8}$ | $2.00 \times 10^{-8}$ | $2.00 \times 10^{-8}$ |
| EDTA | $2.00 \times 10^{-5}$ | $2.00 \times 10^{-5}$ | $2.00 \times 10^{-5}$ |
| Cysteine | 0.00 | $1.00 \times 10^{-5}$ | $2.00 \times 10^{-5}$ |

Note that the solutions were open to atmosphere.

**Table S4. Chemical composition of the exposure medium for marine diatom *T. weissflogii* pre-cultured with cysteine (i.e., Figures 3 & S2). Total concentrations of the chemical components are shown in mol·$L^{-1}$.**

| Treatment | EDTA+Cd | EDTA+Cys+Cd | Cys+Cd |
|---|---|---|---|
| pH | 8.0 | 8.0 | 8.0 |
| $Na^{+}$ | $4.80 \times 10^{-1}$ | $4.80 \times 10^{-1}$ | $4.80 \times 10^{-1}$ |
| $Cl^{-}$ | $5.60 \times 10^{-1}$ | $5.60 \times 10^{-1}$ | $5.60 \times 10^{-1}$ |
| $SO_4^{2-}$ | $2.88 \times 10^{-2}$ | $2.88 \times 10^{-2}$ | $2.88 \times 10^{-2}$ |
| $K^{+}$ | $1.02 \times 10^{-2}$ | $1.02 \times 10^{-2}$ | $1.02 \times 10^{-2}$ |
| $CO_3^{2-}$ | $2.38 \times 10^{-3}$ | $2.38 \times 10^{-3}$ | $2.38 \times 10^{-3}$ |
| $Br^{-}$ | $8.40 \times 10^{-4}$ | $8.40 \times 10^{-4}$ | $8.40 \times 10^{-4}$ |
| $BO_3^{2-}$ | $4.85 \times 10^{-4}$ | $4.85 \times 10^{-4}$ | $4.85 \times 10^{-4}$ |
| $F^{-}$ | $7.15 \times 10^{-5}$ | $7.15 \times 10^{-5}$ | $7.15 \times 10^{-5}$ |
| $Mg^{2+}$ | $5.46 \times 10^{-2}$ | $5.46 \times 10^{-2}$ | $5.46 \times 10^{-2}$ |
| $Ca^{2+}$ | $1.05 \times 10^{-2}$ | $1.05 \times 10^{-2}$ | $1.05 \times 10^{-2}$ |
| $Sr^{2+}$ | $6.38 \times 10^{-5}$ | $6.38 \times 10^{-5}$ | $6.38 \times 10^{-5}$ |
| EDTA | $1.00 \times 10^{-4}$ | $1.00 \times 10^{-4}$ | 0.00 |
| Cysteine | 0.00 | $5.00 \times 10^{-6}$ | $7.70 \times 10^{-5}$ |
| total Cd | $5.00 \times 10^{-8}$ | $5.00 \times 10^{-8}$ | $5.00 \times 10^{-8}$ |

Note that the solutions were closed to atmosphere.

## 4 Rinse solutions

### 4.1 Rinse solutions for the green alga *C. reinhardtii*

Two rinse solutions were used. The first solution, without any ligand (EDTA), was used to rinse the cells harvested from the algal culture to remove extracellular remaining solution (i.e., culture medium and algal debris/metabolites) just before initiating the uptake test. The chemical composition of this rinse solution was the same as the exposure medium to be used but without addition of Cd, EDTA and cysteine.

The second rinse solution, containing EDTA, was used to remove extracellular Cd after the period of Cd uptake, and its composition was the same as the first rinse solution except for the inclusion of 1 mM EDTA (Hassler et al. 2004). Moreover, the 0.05 M $Ca(NO_3)_2$ was replaced by 0.15 M $NaNO_3$ to improve the washing efficiency while keeping a constant ionic strength, since the exchange kinetics between Ca-EDTA and free trace metal ions are slow (Hering and Morel 1989).

### 4.2 Rinse solutions for the marine diatom *T. weissflogii*

Two rinse solutions were used. The AQUIL medium (Sunda et al. 2005) without addition of major nutrients, trace elements, EDTA or vitamins was used to rinse the diatoms harvested from the culture just before initiating the uptake tests. The other wash solution was made following the recipe described by Tang and Morel (2006), and the wash solution contained 100 mM oxalate, 50 mM $Na_2EDTA$, 0.3 M NaCl and 0.01 M KCl at pH 7.0.

## 5 References


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