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Biological uptake of Cd, Se(IV) and Zn by *Chlamydomonas reinhardtii* in response to different phosphate and nitrate additions

Ri-Qing Yu[1,2], Wen-Xiong Wang[1,*]

[1]Department of Biology, Hong Kong University of Science and Technology (HKUST), Clear Water Bay, Kowloon, Hong Kong, SAR

[2]*Present address:* Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803, USA

ABSTRACT: We investigated the influences of different nutrient regimes on the accumulation of Cd, Zn, and Se(IV) in the freshwater green alga *Chlamydomonas reinhardtii* over a 4 h exposure period. After the cells had been acclimated to different ambient P levels from 0.1 to 10.0 µM for 2 d in different media (buffer, basic and simplified uptake media), their intracellular metal concentrations increased by 3.6 to 14× for Cd and 1.6 to 4.0× for Zn. Se uptake was, however, decreased by 7.7 to 44×. Semi-continuous culture experiments further demonstrated that the alga's metal concentrations were enhanced 26× for Cd and 8.0× for Zn, whereas Se accumulation was inhibited 75× at the same medium P levels. The uptake rates in semi-continuous cultures increased by 269× for Cd and 11× for Zn, but Se uptake decreased 92× with increasing P concentration. Medium P additions also significantly increased intracellular partitioning for Cd and Zn, but there was no apparent effect for Se. For P-starved or P-repleted cells, the P-enriched medium dramatically increased the cellular accumulation of Cd and Zn compared with the P-depleted medium. A surge uptake of Cd and Zn occurred in P-starved cells. P starvation also resulted in a remarkable increase in Se accumulation in the P-deplete medium. N enrichment from 5 to 200 µM significantly increased the alga's uptake and intracellular partitioning for Cd and Zn, although the effects were not strictly concentration-dependent. Our study strongly suggests that ambient P enrichment greatly stimulates Cd and Zn accumulation and sequestration in *C. reinhardtii*, presumably due to rapid P uptake and subsequent formation of polyphosphate in the cells, and distinctly inhibits Se uptake possibly due to the competition of similar anionic forms for transport between Se and P.

KEY WORDS: Uptake · Metals · Green alga · Nutrient enrichment

INTRODUCTION

The biogeochemical processes of trace metals in limnetic waters may involve biological recycling, sediment fluxes, oxidation-reduction cycles, and scavenging by particles (Murray 1987). As efficient scavengers, phytoplankton play a critical role in trace metal cycling in the pelagic (Sanders & Riedel 1998). A possible stoichiometric relationship between Cd, Ni, and Zn and nutrient elements in the water column has been proposed for oceans and freshwater lakes (Sigg 1985, Murray 1987). A strong correlation was found between dissolved cadmium and phosphate concentration in lakes (Balistrieri et al. 1992). Reynolds & Hamilton-Taylor (1992) indicated that the distribution of dissolved Zn in a limnetic water column during a diatom bloom was positively correlated with phosphate and silicate, and with atomic C:P:Zn ratios of 106:1:0.034. Based on the potential correlations of trace metals with nutrient concentrations, Morel & Hudson (1985) suggested that the traditional Redfield ratio for marine phytoplankton should be extended to trace metals. However, few studies have tested the interaction between nutrient enrichment and intracellular metal

*Corresponding author. Email: wwang@ust.hk

concentrations in phytoplankton, especially in freshwater species, although this may be important in understanding the stoichiometric mechanisms in lake systems.

Under N-limited conditions, a significant reduction in photosynthetic activity occurs in green algae, including a sharp decline in their chlorophyll content and a rise in carotenoid concentration (Sayed 1998, Hanagata & Dubinsky 1999). When the phytoplankton are under P-limited conditions, the uptake system for phosphate in cells is highly activated. Abrupt exogenous P enrichment results in a rapid P incorporation and storage in intracellular polyphosphates (Kulaev & Vagabov 1983, Wagner & Falkner 2001). Polyphosphate bodies are considered an important pool for the sequestration of heavy metals by freshwater phytoplankton (e.g. Cd, Hg, Pb, and Zn) (Jensen et al. 1982a). Although metal limitation (e.g. of Fe) in phytoplankton may affect the assimilation of nitrate and phosphate (McKay et al. 2001), the effects of nutrient enrichment or starvation on metal uptake by freshwater phytoplankton have received little attention. In marine diatoms, Wang et al. (2001a,b) recently reported that nitrate enrichment significantly increased the uptake of Cd and Zn. Whether the nutrient regimes similarly affect a metal uptake in freshwater green algae is largely unknown.

Lake eutrophication undermines the water quality and the stability of aquatic ecosystems and has become a worldwide concern (Smith et al. 1999, Bennett et al. 2001). In China, over 65% of the lakes are eutrophied, i.e. they are mostly characterized by high phosphate loads (Tang & Xie 2000, Xie & Xie 2002). Excessive nutrient enrichment in lakes not only causes a significant increase in algal biomass, but also leads to considerable stoichiometric changes in organic/inorganic chemical elements, which possibly include an increasing flux of organic matter, anoxia, sulfide formation, and a faster flux or sequestration of trace metals in sediments. Lithner et al. (2000) proposed that high fluxes of metals in a eutrophic lake might partly be explained by a large algal biomass and different geological conditions (e.g. property of suspended particulate matter) and water chemistry (e.g. pH). They showed that eutrophication decreased the concentrations of Cd and Mo in the water, probably due to a large algal biomass and efficient scavenging of metals. Recently, Pickhardt et al. (2002) found that increasing phosphate concentration reduced methylmercury concentrations in freshwater algae and *Daphnia mendotae* feeding on contaminated algae. They hypothesized that increasing algal biomass under high P conditions reduced methylmercury concentration in the algae. However, besides such 'biomass dilution', other mechanisms may also be involved in a possible interaction between eutrophication and contamination (Gunnarsson et al. 1995, Luoma et al. 1998). There is a substantial need to study the interaction between these 2 ecological 'stressors' in freshwater systems.

In this study, we investigated the accumulation of metals in the freshwater green alga *Chlamydomonas reinhardtii* under different conditions of major nutrients, namely, phosphate and nitrate. We considered 3 metals/metalloids: Cd, Se, and Zn; of these, Se and Zn are essential elements, whereas the essentiality of Cd to freshwater phytoplankton has not been demonstrated (as compared to marine diatoms; Cullen et al. 1999). Different nutrient regimes, including short-term nutrient acclimation, semi-continuous cultures, and nutrient starvation of phosphate and nitrate were considered in this study. Metal uptake was quantified as the intracellular concentration and distribution in the algae under different nutrient conditions.

MATERIALS AND METHODS

Alga culture. An axenic culture of *Chlamydomonas reinhardtii*, a euryhaline unicellular green alga, was originally obtained from the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan. This species is found in eutrophic lakes and is abundant during the spring bloom (Olsen et al. 1983). The alga was maintained in an artificial freshwater WC medium (Guillard 1975) at 23.5°C and a light regime of 100 µmol photons m^{-2} s^{-1} with a 14:10 h light:dark (LD) cycle.

Metal uptake under different nutrient regimes. Short-term exposure (4 h) was employed to quantify metal uptake by the algal cells. We used the radiotracers, ^{109}Cd (in 0.1 N HCl), ^{75}Se (as selenite in distilled water), and ^{65}Zn (in 0.1 N HCl), purchased from New England Nuclear, to trace the uptake of stable metals in the cells. The algal cells were exposed to radiotracers and stable metals under different nutrient conditions over 4 h. For all experiments (except the uptake using EDTA buffer, see below), the concentrations of stable metals added were 17.8 nM for Cd, 25.3 nM for Se(IV), and 76.5 nM for Zn. Radioisotope additions were 29.6 kBq l^{-1} (corresponding to 2.4 nM) for ^{109}Cd, 44.4 kBq l^{-1} (corresponding to 11.7 nM) for ^{75}Se, and 55.5 kBq l^{-1} (corresponding to 0.40 nM) for ^{65}Zn. Microliter volumes of 0.5 N Suprapure NaOH were added to the 0.22 µm-filtered deionized water to maintain the pH at 7.0. Stable metals, radiotracers, and macronutrients were equilibrated overnight before the uptake experiments.

All uptake experiments were conducted in 150 ml of 0.2 µm-filtered deionized water held in acid-cleaned polycarbonate bottles at a light intensity of 100 µmol photons m^{-2} s^{-1}, at 23°C. Triplicate polycarbonate

flasks were prepared for each nutrient treatment. The algal cells which had previously been inoculated under different nutrient conditions (described in the following subsection) were collected onto 1 µm polycarbonate membranes, rinsed 3 to 4 times with filtered water, and resuspended in 0.22 µm-filtered deionized water. The cells were then added to 150 ml filtered deionized water containing stable and radioactive metals and different nutrient concentrations to attain an initial cell density of 1×10^5 cells l^{-1}. Cell density was quantified by a hemocytometer. At Time zero (0 h), subsamples of the uptake solution were removed from each bottle for measurements of cell density (0.7 ml) and radioactivity of the total solution (1 ml). At subsequent time intervals (0.5 to 2 h), a 10 ml aliquot was taken each time from each flask, filtered onto a 1 µm polycarbonate membrane, and rinsed 3 times with filtered deionized water. The algae were then washed with 10 ml of a Na_2EDTA solution (100 µM) for 10 min, and further rinsed 3 to 4 times with 0.22 µm-filtered deionized water, after which the radioactivity of the intracellular metals in the cells was counted. The EDTA washing technique can efficiently remove metals bound to the surface of the algal cells (Bates et al. 1982, Errecalde & Campbell 2000). The efficiency of EDTA washing in removing extracellular Se was, however, unknown and was not tested in this study. The radioactivity in the whole cells (both extracellular and intracellular metals) was measured by rinsing the cells 3 times only with the 0.22 µm-filtered deionized water. A control treatment containing the same uptake medium but without cells was used to monitor the precipitation of metals and the potential sorption of metals onto the polycarbonate membrane. Only a negligible percentage of metals was detected in the polycarbonate membrane (<0.9% for Cd and Zn, and <0.6% for Se(IV) in experiments involving N and P additions). The decrease of radioactivity in the water due to uptake by the cells amounted to 5–41, 0.4–35, and 5–67% for Cd, Se, and Zn, respectively, among the different experimental treatments.

Nutrient conditions. The stock nutrient media were prepared with 0.22 µm Nanopure deionized water and were passed through a Chelex ion exchange resin column to remove trace metals and then autoclaved. All the polycarbonate bottles and glassware were immersed in 14% HNO_3 for 4 to 6 h, rinsed by 0.22 µm-deionized water, and autoclaved to reduce contamination before experiments. The handling of glassware (except the 4 h uptake sampling) was carried out near a propane flame to sterilize and thus avoid possible contamination. To acclimate the cells under each nutrient regime (described below), cells in log-phase growth period were filtered from their stock cultures onto a 3 µm polycarbonate membrane to remove the nutrient medium and were inoculated at each nutrient regime at an initial density of 2×10^5 cells l^{-1}. The cells were maintained under the same light regimes, with gyratory shaking at 80 rpm. After nutrient acclimation, the cells were gently filtered onto a 1 µm polycarbonate membrane under a vacuum pressure of ≤10 cm Hg, and were used for the 4 h uptake experiment. Meanwhile, another set of flasks under the same nutrient conditions were used for measurements of cell dry weight. The algal cells were filtered onto a preweighted glass fiber filter, rinsed with 0.22 µm-filtered deionized water, and dried at 80°C overnight.

In accordance with the general characteristics of eutrophication in shallow lakes (Smith et al. 1999, Havens et al. 2001), we chose a concentration range of phosphate (0.1, 1.0, 10.0 µM) and nitrate (5, 40, 200 µM) in our experiments that mimicked the different nutrient conditions in natural lakes (oligotrophic, mesotrophic, and hypertrophic). A WC medium was chosen to manipulate the algal nutrient treatment experiments because the concentrations of phosphate and nitrate in this medium are relatively low. We performed 5 experiments to determine the influence of nutrition regimes on metal uptake in the green alga.

The first 3 experiments examined the effects of a 2 d acclimation (short-term treatment) at different nutrient concentrations on metal uptake by the cells. Late exponentially growing cells were collected by filtration and resuspended in 300 ml of 0.2 µm-filtered deionized water (autoclaved) containing different nutrient additions. After 2 d acclimation, the cells were again collected on a 1 µm polycarbonate membrane and resuspended in 0.2 µm-filtered deionized water. After cell density (cell abundance) counts, metal uptake by the cells was quantified as described in the preceding subsection. Nutrient concentrations were maintained at the same levels as those used during the acclimation period.

In the first uptake experiment, we only used the basic solution (stable, radioactive metals, and phosphate or nitrate in 0.22 µm-filtered deionized water) for cell exposure over a 4 h period. A microscopic check of the alga in the basic solution during the 4 h period indicated that the cells were not growing but were still alive. In the second uptake experiment, a simplified uptake medium was prepared to analyze the differences in uptake between the basic solution and the simplified medium. The simplified medium was a low ionic strength solution, consisting of several major ions: 68.0 µM $CaCl_2$, 81.1 µM $MgSO_4$, and 4 mM KCl (Fortin & Campbell 2000, Macfie & Welbourn 2000). Other constituents, including stable radioactive metals and phosphate, were the same as in the first experiment. In the third experiment, we

separately examined the influences of 2 different phosphate concentrations (0.1 and 10.0 µM) on the free ion uptake of Cd or Zn using an EDTA buffer solution. The concentration of stable Cd or Zn additions was 0.09 and 0.9 µM, respectively. The simplified medium with 1 µM Na_2EDTA, stable Cd or Zn, and phosphate was used as algal uptake solution. Metal speciation in the solutions was calculated by the chemical speciation model MINEQL+ (Schecher & McAvoy 1994), and the free ion concentration was 0.45 nM for Cd^{2+} and 8.1 nM for Zn^{2+}. The fourth experiment was designed to compare the metal uptake of the green alga under semi-continuous cultures at different levels of N or P. Cells in the late exponential growth phase from the same batch were filtered and resuspended in filtered deionized water containing different P (0.1, 1.0, and 10.0 µM) or N (5, 40, and 200 µM) concentrations. Other nutrients were maintained at WC level. When the cells reached mid-exponential growth phase (1 to 2 d), they were transferred to a new medium containing the same P or N concentration. After 4 transfers (6 d), the cells were filtered again and resuspended in the solution for metal uptake measurements. In the fifth experiment, the effects of nutrient (P, N) starvation on metal uptake in the cells were measured. The algal cells in the exponential growth phase were filtered and inoculated in a nutrient-enriched solution (50.0 µM for phosphate, 1 mM for nitrate), or a nutrient-starved solution (no P or N added). Other nutrients were added at WC levels to ensure that the cells were only starved for 1 specific nutrient. The cell density count indicated that the alga did not grow when the cells were starved of P for 3 d and N for 2 d. Thus we starved the cells of P for 3 d and N for 2 d. Both starved and enriched cells were finally filtered and resuspended in either nutrient-depleted or nutrient-enriched, filtered, deionized water for metal uptake experiments.

During the acclimation and uptake periods, changes in P and N concentrations due to cellular uptake were measured by the methods described in Parsons et al. (1985). The results showed that after 2 d acclimation the ambient nutrient concentration decreased by 70 to 80% at the lowest nutrient treatment, whereas the decline was only 10 to 20% at the highest treatment. There was no significant decrease of P or N during the 4 h uptake period.

Radioactivity was determined by a Wallac 1480 NaI(T1) gamma detector (Turku, Finland), at 88 keV for ^{109}Cd, 264 keV for ^{75}Se, and 1115 keV for ^{65}Zn. All analyses were related to appropriate standards and were calibrated for spillover. Counting times were 3 min and were sufficient to yield propagated counting errors <5%.

RESULTS

Over the short-term exposure period (4 h) in the basic medium (no other major ions except the phosphate), the uptake patterns of Cd, Se(IV) and Zn in freshwater alga *Chlamydomonas reinhardtii* displayed a clear difference at different phosphate concentrations (Fig. 1). Ambient P additions from 0.1 to 10.0 µM significantly increased intracellular metal accumulation by 6× for Cd and 2.5× for Zn after 4 h exposure ($p < 0.5$, ANOVA). However, increasing P concentrations obviously reduced Se accumulation by 7.7× in the alga ($p < 0.5$, ANOVA). At 4 h exposure, 62 to 72% of the Se was found in the intracellular compartment and was independent of the different P treatments. The intracellular distribution of Cd and Zn, however, had a positive relationship with elevated P concentrations. The uptake rate was calculated as the slope of the regression between the algal intracellular metal concentration and the time of exposure (0.5 to 4 h, Table 1). Elevated P concentrations remarkably increased the uptake rates. However, the Se uptake rate was conspicuously inhibited by increasing P concentrations.

A similar short-term exposure was performed by using a simplified uptake medium instead of the basic solution to test the comparability of the experimental results of the 2 media. Metal accumulation in the alga

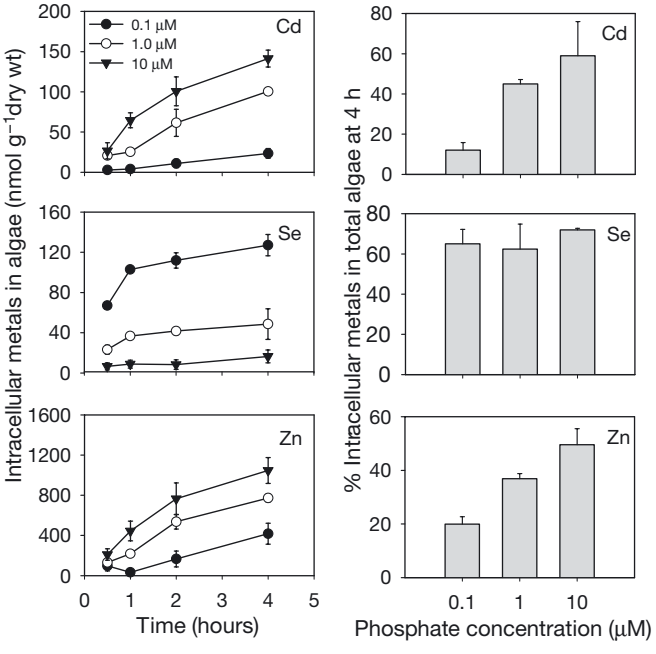

Fig. 1. *Chlamydomonas reinhardtii*. Accumulated intracellular metal concentration (left panels) and intracellular metal distribution (right panels) following short-term acclimation (2 d) in different phosphate concentrations with a basic uptake medium over 4 h. Values are means ± SD (n = 3)

Table 1. *Chlamydomonas reinhardtii*. Calculated uptake rates (nmol g^{-1} h^{-1}) of Cd, Se and Zn under different P and N regimes. Mean ± SD (n = 3). Statistically significant influence of nutrient treatments: *p < 0.05; **p < 0.01; ***p < 0.001 (1-way ANOVA and *t*-test)

Nutrient treatment (μM)	Uptake rate (nmol g^{-1} h^{-1}) Cd	Se	Zn
Phosphate experiments			
2 d acclimation (basic uptake medium)			
0.1 P	6.076 ± 2.615	14.18 ± 2.546	103.7 ± 31.14
1.0 P	23.77 ± 1.317	6.206 ± 3.293	186.3 ± 12.40
10.0 P	30.60 ± 8.264	2.672 ± 0.811	228.2 ± 100.9
Semi-continuous culture			
0.1 P	0.133 ± 0.089**	166.4 ± 52.47**	19.68 ± 4.725**
1.0 P	7.232 ± 5.969**	39.02 ± 8.308**	78.62 ± 59.44**
10.0 P	34.92 ± 6.067**	1.798 ± 0.836**	217.5 ± 44.56**
Starvation			
–P/–P	15.81 ± 0.044*	5.294 ± 2.495	39.70 ± 30.98
–P/+P	68.14 ± 36.52*	0.557 ± 0.013	504.8 ± 307.4
+P/–P	8.611 ± 0.907*	3.571 ± 0.659	115.1 ± 31.90
+P/+P	47.30 ± 23.38*	0.494 ± 0.266	264.1 ± 109.0
Nitrate experiments			
2 d acclimation			
5.0 N	7.313 ± 1.868***	16.88 ± 2.736	8.411 ± 7.035**
40.0 N	4.875 ± 1.797***	19.04 ± 6.003	1.728 ± 1.346**
200.0 N	18.14 ± 0.365***	13.20 ± 2.634	73.22 ± 26.49**
Semi-continuous culture			
5.0 N	2.188 ± 0.898**	20.50 ± 2.292*	8.411 ± 0.015**
40.0 N	5.302 ± 0.952**	36.89 ± 7.257*	18.12 ± 8.273**
200.0 N	7.775 ± 0.507**	27.81 ± 2.191*	52.90 ± 6.132**
Starvation			
–N/–N	5.774 ± 5.124	19.12 ± 12.10	28.98 ± 23.86
+N/+N	12.19 ± 3.656	17.54 ± 7.016	41.99 ± 14.79

approximately followed a linear trend over time in the simplified exposure medium (Fig. 2). With a rise in the medium P level from 0.1 to 10.0 μM, intracellular metal concentrations increased by 14× and 4× for Cd and Zn, respectively, at 4 h (Cd, $p < 0.001$; Zn, $p < 0.01$; ANOVA), but substantially decreased Se accumulation by 44× ($p < 0.001$, ANOVA). A similar positive relationship between intracellular metal distribution and ambient P enrichment was also found for Cd and Zn.

Metal uptake was also quantified in the buffer solution (using EDTA) to specifically examine the effects of nutrients on the free ion uptake of metals (Fig. 3). When the medium P concentration increased from 0.1 to 10.0 μM, intracellular Cd concentration increased by 3.2× ($p < 0.01$, *t*-test) and Zn uptake increased by 1.6× ($p < 0.05$, *t*-test). The uptake rates for Cd and Zn also increased by 2.6× (from 0.267 to 0.703 nmol g^{-1} h^{-1}) and 2.4× (from 8.411 to 20.38 nmol g^{-1} h^{-1}), respectively (significant only for Cd, $p < 0.01$, *t*-test). There was no obvious variation in intracellular Cd and Zn distribution. However, when compared with the above 2 experiments (basic and simplified medium), the intracellular concentrations of Cd and Zn in the 10.0 μM P treatment decreased considerably, even though the total ambient metal concentrations used in this experiment were much higher.

When the green alga was maintained in semi-continuous culture, its metal uptake was similarly affected by different P regimes (Fig. 4). Intracellular concentrations increased by 26.2× for Cd and by 8.0× for Zn ($p < 0.01$, ANOVA), whereas Se accumulation was inhibited by 75.2× ($p < 0.001$, ANOVA). The uptake rates for Cd and Zn increased by 269× (from 0.133 to 34.92 nmol g^{-1} h^{-1}) and by 11× (from 19.68 to 217.5 nmol g^{-1} h^{-1}) ($p < 0.01$, ANOVA), respectively (Table 1). For Se, the uptake rate decreased by 92× (from 166.4 to 1.798 nmol g^{-1} h^{-1}; $p < 0.01$, ANOVA). Intracellular distribution of Cd and Zn greatly increased, whereas intra-

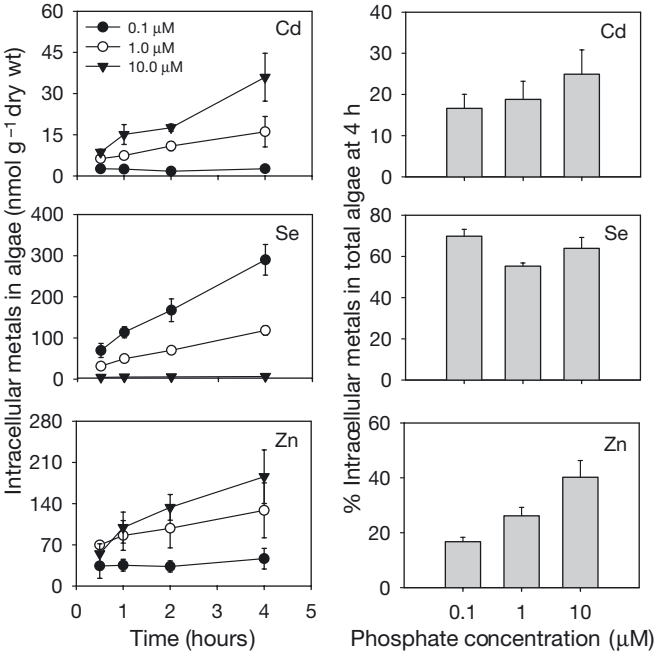

Fig. 2. *Chlamydomonas reinhardtii*. Accumulated intracellular metal concentration (left panels) and intracellular metal distribution (right panels) following short-term acclimation (2 d) in different phosphate concentrations with a simplified uptake medium over 4 h. Values are means ± SD (n = 3)

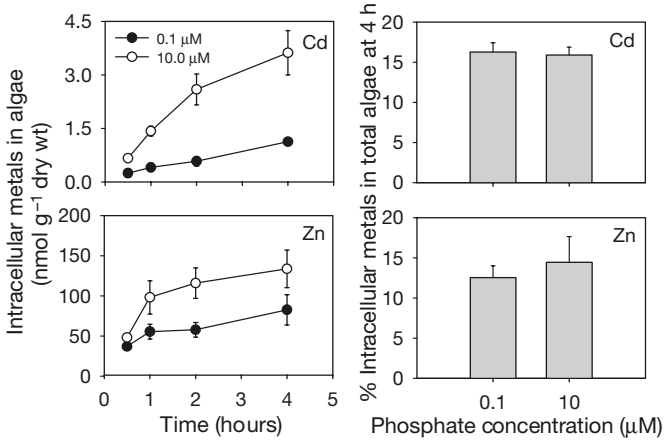

Fig. 3. *Chlamydomonas reinhardtii*. Accumulated intracellular metal concentration (left panels) and intracellular metal distribution (right panels) following short-term acclimation (2 d) in different phosphate concentrations with EDTA buffer solution over 4 h.Values are means ± SD (n = 3)

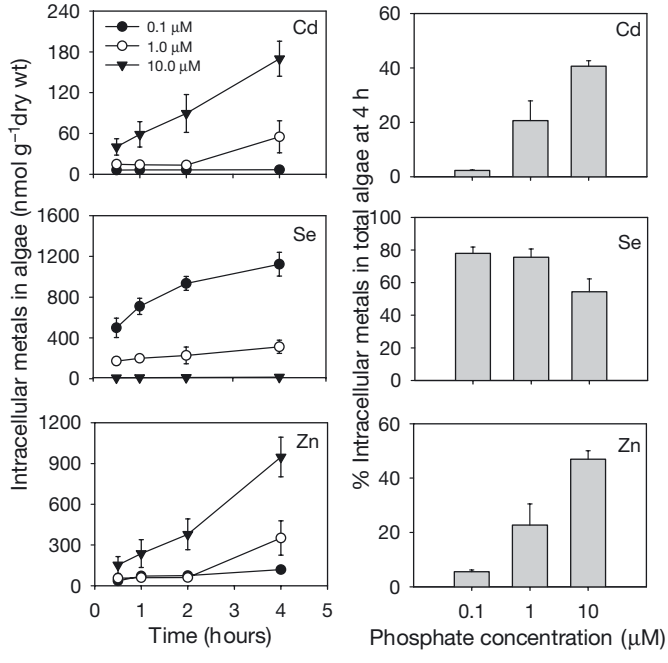

Fig. 4. *Chlamydomonas reinhardtii*. Accumulated intracellular metal concentration (left panels) and intracellular metal distribution (right panels) following semi-continuous culture in different phosphate concentrations over 4 h. Values are means ± SD (n = 3)

cellular Se distribution decreased, with increasing P concentration (for Cd and Zn: $p < 0.001$; for Se: $p < 0.05$, ANOVA).

Phosphate starvation had pronounced effects on metal uptake of the algae (Fig. 5). Cd accumulation was 2.1× lower in P-starved than in P-enriched cells in

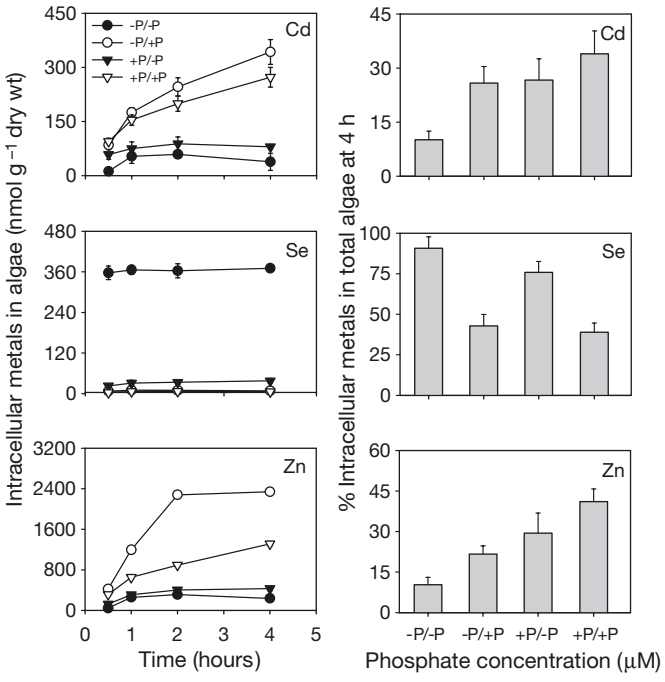

Fig. 5. *Chlamydomonas reinhardtii*. Accumulated intracellular metal concentration (left panels) and intracellular metal distribution (right panels) in P-enriched and P-depleted algae following exposure to P-enriched and P-depleted medium over 4 h. Values are means ± SD (n = 3). –P/–P: P-starved cells in P-depleted medium; –P/+P: P-starved cells in P-enriched medium; +P/–P: P-enriched cells in P-depleted medium; +P/+P: P-enriched cells in P-enriched medium

the P-depleted medium after 4 h exposure ($p < 0.05$, *t*-test). In contrast, there was a 1.3× increase in the starved cells compared to enriched cells in the P-enriched medium. Different exposure media (e.g. P-enriched or P-depleted) induced more obvious effects on metal uptake. Cd intracellular concentrations of P-starved cells increased by 9.0× in P-enriched compared to P-depleted medium, and concentrations of P-enriched cells increased by 3.4× when the medium shifted from P-depleted to P-enriched status ($p < 0.05$, *t*-test), indicating surge uptake in the P-enriched medium. Similarly, the Cd uptake rates of P-starved cells were 4.3× higher in the enriched than in the depleted medium. For P-enriched cells, the uptake rate increased by 5.5× (from 8.611 to 47.30 nmol g^{-1} h^{-1}) in P-enriched compared to P-depleted medium.

For Zn, when both the P-starved cells and P-enriched cells were resuspended in the P-depleted medium, no significant change of metal accumulation at 4 h was observed. However, the P-enriched medium distinctly increased the Zn accumulation by 9.9× for P-starved cells and by 3.1× for P-enriched cells compared to those of the P-depleted medium ($p < 0.05$, *t*-test). Similarly, the uptake rate increased by 12.7× for P-

starved cells and 2.3× for P-enriched cells in the P-enriched medium compared to the P-depleted medium. P-starved cells accumulated a much higher Se concentration than P-enriched cells in the P-depleted medium. The Se concentration increased by 45× for P-starved cells and by 7× for P-enriched cells in the P-depleted medium compared to those in the P-enriched medium ($p < 0.01$, *t*-test). About 10–26% cellular Cd and 10–22% cellular Zn were associated with the intracellular compartment in P-starved cells at 4 h exposure compared to 27–34% cellular Cd and 29–41% cellular Zn in the intracellular compartment in P-enriched cells. For both P-starved and P-enriched cells in the P-depleted medium, 76–91% of the Se was located in the intracellular pool, whereas only 39–43% of Se was found in this compartment in the P-enriched medium.

An increase in nitrate concentrations from 5 to 200 µM significantly enhanced the accumulation of Cd (by 1.8×) and Zn (by 7.0×) in green algal cells previously acclimated to nitrate for 2 d (Fig. 6; $p < 0.001$). Uptake rates also increased from 7.313 to 18.14 nmol g^{-1} h^{-1} for Cd and from 8.411 to 73.22 nmol g^{-1} h^{-1} for Zn. However, Cd and Zn accumulation was fairly comparable between 5 and 40 µM N. Among the different N levels, there was also significant variation in intracellular distribution for Cd and Zn ($p < 0.01$, ANOVA),

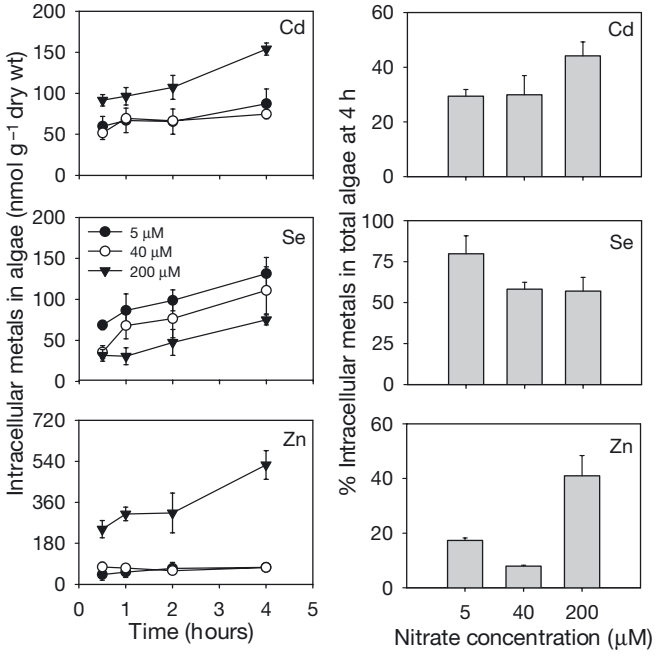

Fig. 6. *Chlamydomonas reinhardtii*. Accumulated intracellular metal concentration (left panels) and intracellular metal distribution (right panels) following short-term acclimation (2 d) in different nitrate concentrations over 4 h. Values are means ± SD (n = 3)

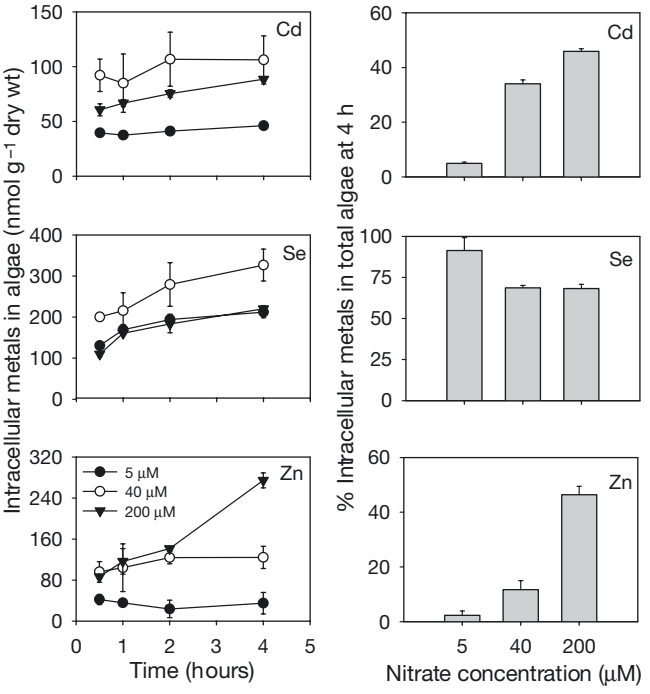

Fig. 7. *Chlamydomonas reinhardtii*. Accumulated intracellular metal concentration (left panels) and intracellular metal distribution (right panels) following semi-continuous culture in different nitrate concentrations over 4 h. Values are means ± SD (n =3)

which were much higher at the highest nitrate concentration examined (200 µM). Nitrate addition generally decreased both Se accumulation and intracellular distribution, and the uptake rate decreased from 16.88 to 13.20 nmol g^{-1} h^{-1} when the ambient N concentration increased from 5 to 200 µM.

A similar influence of N levels on Cd and Zn uptake was further confirmed by the semi-continuous experiments (Fig. 7). When ambient N concentrations increased from 5 to 200 µM, Cd and Zn accumulation at 4 h increased by 1.9× and 7.8× ($p < 0.01$, ANOVA), and uptake rates by 3.6× and 6.3×, respectively. For Cd, the effect also was not concentration-dependent. Cd and Zn intracellular distribution increased with increasing N levels ($p < 0.001$, ANOVA). Although the Se accumulation between 5 and 200 µM was fairly comparable, a significant difference in accumulation and uptake rate was observed among the 3 N treatments.

Under N starvation, Cd and Zn concentrations of the algal cells were 1.8× and 1.9× lower than in N-enriched cells after 4 h exposure, whereas the intracellular partitioning was much higher (Fig. 8). In contrast, nitrate starvation did not significantly affect either accumulation or intracellular distribution of Se in the cells.

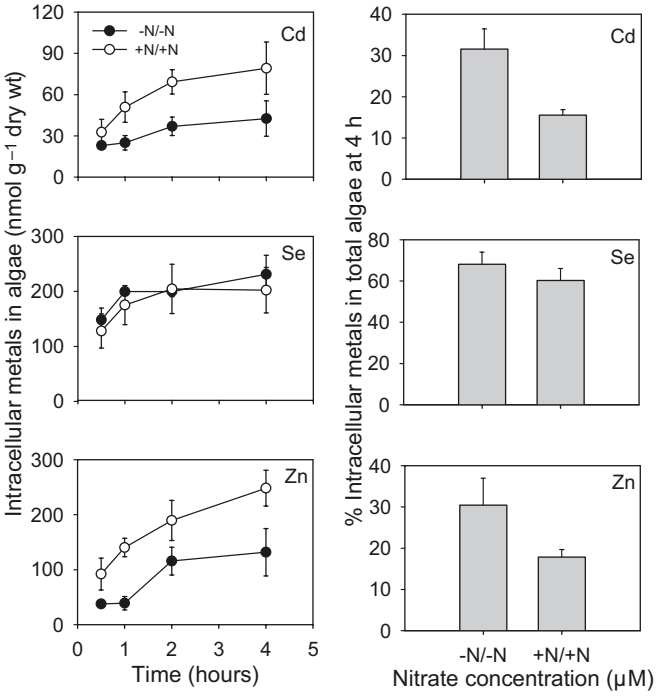

Fig. 8. *Chlamydomonas reinhardtii*. Accumulated intracellular metal concentration (left panels) and intracellular metal distribution (right panels) in N-enriched and N-depleted alga following exposure in N-enriched and N-depleted medium over 4 h. Values are means ± SD (n = 3). –N/–N: N-starved cells in N-depleted medium; +N/+N: N-enriched cells in N-enriched medium

DISCUSSION

Short-term exposure was employed to examine algal metal uptake in order to minimize the effects of efflux, metal complexation by algal exudates, and variation in cell size and biomass (Wang et al. 2001a,b, Slaveykova & Wilkinson 2002). The cells were acclimated to different nutrient regimes using short-term acclimation (2 d), semi-continuous culture, or starvation (Olsen et al. 1983, Wang et al. 2001a,b). In general, we found that the metal uptake by *Chlamydomonas reinhardtii* displayed a 2-phase process, comprising rapid initial surface sorption and a subsequent slow transport into the intracellular compartment. Using different media (basic, simplified, and EDTA buffer solution), we demonstrated that P enrichment considerably increased Cd and Zn uptake and inhibited Se accumulation in the freshwater green alga *C. reinhardtii*. Semi-continuous culture experiments with a longer nutrient acclimation period further confirmed such nutrient influences. We are not aware of previous studies concerning the influences of P addition on Cd and Zn uptake in freshwater green algae. Pettersson et al. (1985) revealed that P concentrations in the medium (35 and 180 µM) affected Al accumulation patterns in the cyanobacterium *Anabaena cylindrica*. After inoculating the cells in a P-enriched medium (4 or 24 h), more Al bound to the polyphosphate granules and the cell walls. In another cyanobacterium, *Nostoc muscorum*, Singh et al. (1992) reported that a 2 to 3× increase in medium P concentration promoted Ni accumulation after culturing cells for 6 d. However, the P concentration (58 to 174 µM) used in their experiments was relatively high compared to realistic P levels in natural waters. Recent studies also suggested that the metal-binding abilities of the cyanophyceaen *Lyngbya taylorii* for Cd, Pb, Ni, and Zn could be improved by phosphorylation of the alga, which may be important in wastewater purification using this group of algae (Klimmek et al. 2001).

Metalloid Se may generally be taken up through the anionic channel (e.g. sulphate or phosphate analogy) (Simkiss & Taylor 1995). By examining selenite uptake in *Chlamydomonas reinhardtii*, Riedel & Sanders (1996) showed that Se accumulation significantly increased in cells with reduced medium P concentrations. Sanders & Riedel (1998) further suggested that the accumulation of elements with anionic forms (e.g. SeO_3, CrO_4, and AsO_3) is usually related to competition for uptake with a similar nutrient ion (eg. Se-S, Cr-S, As-P). Our study was consistent with these previous results insofar as Se uptake by the freshwater green alga was competitively inhibited by high P concentration in the ambient water.

One likely explanation for the increased metal uptake is that P enrichment increases the binding capacity of polyphosphate bodies (in numbers and volume), which in turn facilitates metal accumulation in the cells. Several studies have documented that many metals (e.g. Pb, Mg, Zn, Cd, Sr, Co, Hg, Ni, Cu) can be sequestered in polyphosphate bodies by cyanobacteria and eukaryotic algae (Sicko-Goad & Stoermer 1979, Baxter & Jensen 1980, Jensen et al. 1982a,b, Rachlin et al. 1982). The presence of polyphosphate bodies in bacteria, cyanobacteria, and freshwater unicellular algae has been widely reported (Harold 1966, Baxter & Jensen 1980, Sicko-Goad & Lazinsky 1986, Goldberg et al. 2001). P limitation is a typical phenomenon in freshwater systems (Schindler 1977). In environments with fluctuating P concentrations, accumulating P at a faster rate is probably one of the most fundamental evolutionary adaptations by freshwater phytoplankton (Scavia et al. 1984, Sommer 1984, Sakshaug & Olsen 1986). When the availability of exogenous phosphorus exceeds the metabolism requirement of cells, polyphosphate bodies can be quickly formed (Kulaev 1979, Cembella et al. 1984, Nissen et al. 1987). Polyphosphate bodies are located in the intracellular sector, primarily functioning as a phosphate reserve and an osmotically inert sink for metabolic cations (eg. K^+, Na^+, Ca^{2+}, Mg^{2+}), and also acting as a detoxification

mechanism for heavy metals (Sicko 1974, Peverly et al. 1978, Sicko-Goad & Lazinsky 1986). Jensen et al. (1982a) suggested that polyphosphate in the cyanobacterium *Plectonema boryanum* could efficiently bind positive ions (Cd, Cu, Pb and Hg) mainly because of its strong negative surface charge. Cells of the green alga *Chlamydomonas eugametos* were also found to contain polyphosphate in the form of vacuole-like organelles in the cytoplasm (Coleman 1978, Siderius et al. 1996). Jensen et al. (1982b) indicated that Pb and Zn were concentrated in cell sectors with polyphosphate bodies in *Chlorella saccharophila*.

With increasing ambient P concentrations, the fractions in Cd and Zn in the intracellular pool of algal cells also increased. In the EDTA buffer solution, there was no obvious variation of Cd and Zn intracellular distribution between the 0.1 and 10.0 µM P treatments. Medium EDTA can compete strongly for free Cd and Zn ions with cellular surface absorption and the binding sites in intracellular polyphosphate bodies, and thus may weaken the influence of ambient P regimes on metal subcellular distribution. After culturing *Scenedesmus subspicatus* in ambient Zn concentrations from 10^{-9} to 10^{-16} M for 5 d, Knauer et al. (1997) recorded that the total and intracellular Zn^{2+} content were almost the same. However, with increasing free Zn^{2+} concentration from 10^{-9} to 10^{-5} M, the amount of zinc absorbed to the cell walls increased from 5 to 80% of the total cellular content. Our short-term exposure (4 h) experiments found that the fractions of metal associated with the intracellular pool were rather variable (2.3 to 58.9% for Cd, 54.4 to 78.0% for Se, and 5.5 to 49.5% for Zn) at different medium P concentrations from 0.1 to 10.0 µM. Reynolds & Hamilton-Taylor (1992) showed that during the development of a spring bloom in Windermere Lake, England, the Zn concentration in the diatom *Asterionella* spp. increased, reaching up to 50% of that in solution at the peak of diatom bloom, but no notable changes in P concentration were documented in their study. Nagel et al. (1996) investigated the subcellular distribution of Cd^{2+} in a Cd-tolerant mutant of the cell-wall-deficient *Chlamydomonas reinhardtii*. Their study indicated that cytosol contained only about 10% of the incorporated Cd^{2+}; the major proportion was in the purified chloroplasts (>50%).

In our starvation experiments, the addition of phosphate to the uptake medium dramatically increased the cellular accumulation of Cd and Zn in both P-starved and P-enriched cells compared with those cells in the P-depleted medium. These data strongly indicated that P played a critical role in stimulating Cd and Zn uptake in *Chlamydomonas reinhardtii*. In the P-enriched medium, P-starved cells accumulated a higher concentration of Cd and Zn than P-enriched cells, indicating a surge uptake of Cd and Zn for these P-starved cells. Using magnetic resonance spectroscopy, Hebeler et al. (1992) showed that most of the inorganic P given to P-starved *C. reinhardtii* cells was taken up and accumulated as polyphosphate. Watanabe et al. (1989) found that cellular P concentrations in the marine flagellate *Heterosigma akashiwo* rapidly increased from 0 to 11.4 fmol $cell^{-1}$ in 2 h and further to 24.7 fmol $cell^{-1}$ in 6 h when P-depleted cells were enriched with 4.5 µM P. Considering the simultaneous excretion of Zn, Cu, K, Mg, Ca, and uptake of large quantities of Mn by the cells, they further suggested that metals needed for polyphosphate synthesis may be highly specific among different algal species, and that charge-balancing mechanisms may exist in *H. akashiwo* cells. In marine diatoms, P starvation resulted in increased Zn accumulation (Wang et al. 2001a), but Cd uptake was not influenced by P starvation (Wang et al. 2001b). When the cells of *C. eugametos* were starved of phosphate, Siderius et al. (1996) recorded that they accumulated ^{32}P much faster than control cells, similar to the phenomenon of metal surge uptake in P-starved cells in this study. They found that most ^{32}P was in a metabolically inactive storage form with less than 5% presented in soluble molecules. Based on our results for *C. reinhardtii*, kinetic uptake of Cd and Zn by P-starved cells in P-enriched media was strongly correlated with the transient change of cellular P. Rapid P uptake and subsequent formation of polyphosphate in the cells greatly stimulated Cd and Zn accumulation in the green alga. For Se, P-starved cells in the P-depleted medium accumulated the highest Se concentration possibly due to the lowest antagonistic competition with P (Sanders & Riedel 1998).

N enrichment significantly increased the algal uptake and intracellular partitioning of Cd and Zn, although such influences were less pronounced than those of P additions. A considerable increase in metal accumulation occurred only at the highest N level (200 µM) in the short-term acclimation experiments. The obvious effects were also not consistent for Cd and Se in semi-continuous experiments. The effects of N enrichment on metal uptake in *Chlamydomonas reinhardtii* were therefore highly metal-specific and nutrient status-specific. For freshwater green algae, there are few studies concerning the influences of N enrichment on metal uptake in cells, presumably because nitrate is generally not considered a nutrient-limiting factor in the system. In marine algae, recent studies demonstrated that N enrichments substantially enhance the accumulation of Cd and Zn by marine diatoms (Wang et al. 2001a,b). Rijstenbil et al. (1998) also showed that N-addition significantly increased cellular Cu, Mn, and Zn accumulation in the diatom *Thalassiosira pseudonana* during a 14 d batch culture. Increasing glutathione synthesis was proposed to

account for the enhanced Zn uptake by the diatoms. However, the mechanisms of the interaction between metal uptake and N enrichment in freshwater green algae are still not clear.

Eutrophication in lake systems may significantly increase the flux of suspended particulate matters and the possibility of anoxic events. Lithner et al. (2000) showed that eutrophication decreases the concentration of Cd and Mo in the water column due to large algal biomass and efficient scavenging of metals. Consequently, the residence time of Zn and Cd is extremely short compared to oligotrophic lakes in Sweden. Based on our experimental results, the much higher uptake rate and cellular accumulation of Cd and Zn after P enrichment suggests that *Chlamydomonas reinhardtii* can transfer more metals from the ambient water to its cells, and thus reduce their concentrations and retention times in the water column. Increasing sequestration in the algal cells can accelerate metal sedimentation and flux to the lake bottom. For Se, however, lower uptake and cellular accumulation may increase its concentration and retention time in the water column. In addition, Twiss & Nalewajko (1992) showed that polyphosphate in *Scenedesmus acutus* played a passive role in protecting cells from Cu toxicity. A higher cellular P content alleviated photosynthesis inhibition during Cu exposure. Whether the polyphosphate acts as a sink for metals and thus prevents or reduces metal toxicity needs to be further tested.

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