Supporting information

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Cd tolerance and accumulation in the aquatic macrophyte, *Chara australis*: Potential use for Charophytes in phytoremediation.

Pages: 8

Figures: 1

Tables: 1

**Detailed Experimental Section:**

*Composition of Growth Media*

The media in which the algae were cultured, B&B solution, consisted of 0.1 mM $CaCl_2$, 0.017 mM $KH_2PO_4$, 0.1 mM $MgSO_4$, 0.5 mM $NaHCO_3$, 0.2 mM $NaNO_3$, 1.0 mM NaCl, and 0.05 mM $K_2SO_4$. The medium in which experiments were performed contained 1 mM NaCl, 0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 µM germanium (IV) oxide (to discourage diatom growth), and 5 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, adjusted to pH 5 with 1 M NaOH .

*Source of chemicals.*

Unless otherwise noted, all chemical reagents were from Sigma-Aldrich Corp (St. Louis, MO).

In B&B solution: $CaCl_2$ (Fisher Scientific, Fair Lawn, NJ), $MgSO_4$ (Mallinckrodt Chemicals, Phillipsburg, NJ), 0.5 mM $NaHCO_3$, $NaNO_3$ (Fisher Scientific, Fair Lawn, NJ), $K_2SO_4$ (Mallinckrodt Baker Inc., Phillipsburg, NJ). In the experimental growth solution, KCl (J.T. Baker Chemical Co., Phillipsburg, NJ), $CaCl_2$ (Fisher Scientific, Fair Lawn, NJ), MES buffer, (J.T. Baker Chemical Co., Phillipsburg, NJ).

For cadmium source and standards, Cd was added as nitrate (E.S.P. Chemicals, Inc., Tucson, AZ), except in Expt 3, Cd localization, in which $CdCl_2$ from Mallinckrodt Baker Inc., Phillipsburg, NJ.

For Cd localization, glacial acetic acid (J.T. Baker Chemical Co., Phillipsburg, NJ). For extraction of Cd, $HNO_3$ (TraceSelect®, ≥ 69.0%, Sigma). For extraction of chlorophyll, acetone (J.T. Baker Chemical Co., Phillipsburg, NJ).

**Measurement of Cd.** Cd was measured using a Thermo Scientific X-Series 2 inductively coupled plasma quadrupole mass spectrometer (ICP-MS). Cd standards were prepared in 2% nitric acid (sample background) at 0.01, 0.1, 1, 10, 100, and 1000 µg metal $L^{-1}$ using an ICP-MS standard metal mix (BDH Aristar). Operating conditions of the ICP-MS system were RF power = 1400 W; nebulizer gas flow rate = 0.9 L/min; cooling gas flow rate = 13.0 L/min; auxilliary gas flow rate = 0.70 L/min; mass resolution, m/delta m = 125; Scanning mode/s = survey and peak jumping. The concentration of Cd in each sample was measured by the instrument in triplicate. Standard deviation for each sample was less than 5%.

***Quality Control.*** A blank was prepared by using chemical reagents alone (with no plant or soil sample) following the same extraction procedure described above. Cd concentrations in blank were below detection limits. To ensure the reproducibility of Cd concentrations in samples, two samples whose Cd concentrations were previously measured using ICP-MS, were measured later the same day. Relative percent difference (RPD) between the two measurements was calculated as $\%\ Difference = 100 * \left| \frac{x_1 - x_2}{(x_1 + x_2)/2} \right|$.

The average RPD±SE for the 2 samples re-injected on the same day was 3±1.5 %. Three samples, whose concentrations were previously measured by ICP-MS, were re-injected the following day, and RPD±SE was determined to be 8.5±0.7 %.

***Data Analysis.*** Data for common treatments were combined and averaged. In the growth studies, initial individual explant WW (grams), rhizoid lengths (cm), and branch numbers were recorded. The percent growth rate per day (%GR) was calculated as $100 * \left[ \frac{wet\ weight_{final} - wet\ weight_{initial}}{wet\ weight_{initial}} \right] \div days$. Tolerance indices (TI) were calculated as

$100 * \left[ \frac{rhizoid\ length_{Cd-exposed}}{rhizoid\ length_{Control}} \right]$ [20]. TI of control plants was set equal to 100. Translocation factor (TF) was calculated as $\left[ \frac{concentration\ Cd_{shoots}}{concentration\ Cd_{rhizoids}} \right]$ [21]. Bioconcentration factor (BCF) was calculated as $\left[ \frac{concentration\ Cd_{shoots}}{total\ concentration\ Cd_{soil}} \right]$ [22].

***Statistical Analysis*.** Analysis of variance (one way ANOVA) using SPSS PASW 18 computer software was performed to test the effect of Cd on growth and accumulation in plant tissues. Post hoc comparisons were made using Games-Howell post hoc statistical tests. Significance was based on a p=0.05 level. Bioconcentration and translocation factors were compared to the critical hyperaccumulator value of 1.0 using Student's t-tests in Microsoft Office Excel 2007.

**Glutathione Determination.** Explants were planted in soil with up to 8 added mg Cd $(kg\ soil)^{-1}$ for 97 days, as described for Experiment 1. One to two explants were removed from soil and rinsed with nanopure water deionized using a Nanopure Diamond Ultrapure water system (Barnstead International, Dubuque, IA). Since drying would destroy glutathione, WWs were recorded, and DWs were estimated using a conversion factor determined by averaging the DW/WW ratios of 20 plants from previous experiments. Sample extraction and derivatization procedure were adapted from Guan et al. [23]. Plants were hand-homogenized using a Dounce tissue grinder and Teflon pestle in nanopure water (4 mL $(g\ WW)^{-1}$). Homogenates were filtered using a 45 µm syringe filter tips (Corning International, Corning, NY) and 3 replicate 125 µL aliquots were assayed. Glutathione reduced ethyl ester (GEE, 95% TLC grade, 0.3 µM (100 ppb)), was added as the internal standard for the thiol (GSH) with 4.5 µM (1 ppm) of L-cystine-1, 1-$^{13}C_2$ for the internal standard for the disulfide (GSSG). 2 mM Ellman's reagent (ER, 5,5'-

dithiobis-(2-nitrobenzoic acid)) (Thermo Scientific, Rockford, IL), used to derivatize the thiols, was prepared in 10 mM phosphate buffer prepared using $K_2HPO_4$ (Aldrich) in 100 mL nanopure water. The pH was adjusted to 7.4 using 0.1 M hydrochloric acid (HCl). 250 µL was added to the homogenates, for a final concentration of 1.28 mM ER. Homogenates with ER were incubated at room temperature for 30 min, after which 15 µL 5%-sulfosalicylic acid was added. The sample mixture was centrifuged for 10 min at 13,400 rpm ($12 \times 10^4$ g) using a Mini Spin eppendorf centrifuge to remove precipitated proteins. Samples were analyzed by LC-MS using a LCQ Advantage ion trap mass spectrometer equipped with an electrospray ionization source operated in positive mode (Thermo Fisher Scientific, San Jose, CA), and a C-18 Betabasic column (100 mm×2.1 mm) (Thermo Hypersil-Keystone, Bellefonte, PA). Flow rate was 200 µL $min^{-1}$, column oven temperature was 30 $^{o}$C, and injection volume was 10 µL. The separation was performed using a step gradient of 96.7% nanopure water (with 0.3% formic acid), 3% methanol (Solvent A), and 50% nanopure water (with 0.3% formic acid), 49.7% methanol (Solvent B). LC-MS grade methanol was purchased from Burdick and Jackson (Muskegon, MI). Solvent A was run isocratically for the first 3 min, then switched to solvent B at 4 min, and held for 8 min. The solvent composition was brought back to solvent A, and held for 10 min to equilibrate the column. The electrospray voltage was held at 4 kV, and the capillary temperature was set to 350°C. Selective reaction monitoring was set to simultaneously monitor ions with m/z of the GSH-ER derivative (505→357, 375) and GEE-ER derivative (533→385, 403), along with the disulfides, L-Cystine-1,1 $^{13}C_2$ (213→152, 195) and GSSG (613→345, 483). MS/MS collision energy was set to 30% (normalized) at 0.250 activation energy (the radio frequency used to fragment ions). GSH and GSSG levels in the samples were quantified using a calibration curve ranging from 50 to 500 ng $mL^{-1}$ for GSH and GSSG, with internal standard concentrations

of 100 ng $mL^{-1}$ for GEE and 1000 ng $mL^{-1}$ for L-Cystine-1,1 $^{13}C_2$. The GEE and L-cystine-1, 1-$^{13}C_2$ internal standards were used to correct for the variabilities in HPLC injection and ionization. Standard mixtures of reduced L-glutathione (GSH) and oxidized L-glutathione (GSSG) were prepared using 10 µg $mL^{-1}$ GSH and GSSG stock solutions in water. The same derivatization method that was applied to sample homogenates was used for standard preparation. The detection limits for GSH-ER and GSSG were 2 and 10 ng $mL^{-1}$, respectively. The method detection limit for this method was determined based on three times the signal-to-noise ratio.

**Table S1. *Chara australis* growth responses to Cd exposure (Experiment 4).** Plants were cultured in soil containing up to 25 mg added Cd (kg soil)$^{-1}$ for 49 d (Expt. 4A) or 35 mg added (kg soil)$^{-1}$ (Expt. 4B) for 54 d. Data are mean ± SE of 4 to 11 plants. The background concentration of Cd in unspiked soil was determined to be 0.49±0.12 mg Cd (kg DW)$^{-1}$. Significant differences are based on one way ANOVAs followed by Games-Howell post hoc comparisons for % GR, TI, and BN. Scheffe post hoc comparisons were performed to compare rhizoid lengths ($p<0.05$). In the rhizoid length column, letters represent significant differences. "**A**" signifies significant differences from 5 mg added Cd (kg soil)$^{-1}$, "**B**" from 25 mg added Cd (kg soil)$^{-1}$, "**C**" from 35 mg added Cd (kg soil)$^{-1}$. In the BN column in Expt. 4B, the superscript "**D**" represents a significant difference between the BN of plants cultured in 35 mg added Cd (kg soil)$^{-1}$ to Expt. 4B controls cultured without added Cd.

| **Added Cd mg (kg soil)$^{-1}$** | **Rhizoid Length (cm)** | **Branch Number** | **Shoot DW (g)** | **Rhizoid DW (g)** |
|---|---|---|---|---|
| **Experiment 4A: Incubation up to 25 mg added Cd (kg soil)$^{-1}$ for 49 days** | | | | |
| 0 | 2.9±0.6 | 3.6±0.6 | 0.0018±0.002 | 0.001±0.0003 |
| 5 | 2.4±0.4 | 2.8±0.7 | 0.017±0.004 | 0.002±0.0009 |
| 8 | 3.1±0.4 | 2.8±0.7 | 0.019±0.003 | 0.002±0.0007 |
| 15 | 1.5±0.4$^{ABC}$ | 2.5±0.5 | 0.011±0.002 | 0.0004±8E-05 |
| 25 | 1.6±0.3 | 3.0±0.5 | 0.016±0.003 | 0.0006±0.0001 |
| **Experiment 4B: Incubation in 0 or 35 mg added Cd (kg soil)$^{-1}$ for 54 days** | | | | |
| 0 | 5.1±0.5 | 5.0±0.5 | 0.03±0.003 | 0.005±0.001 |
| 35 | 4.6±0.6 | 1.3±0.1$^{D}$ | 0.009±0.001 | 0.0015±0.001 |

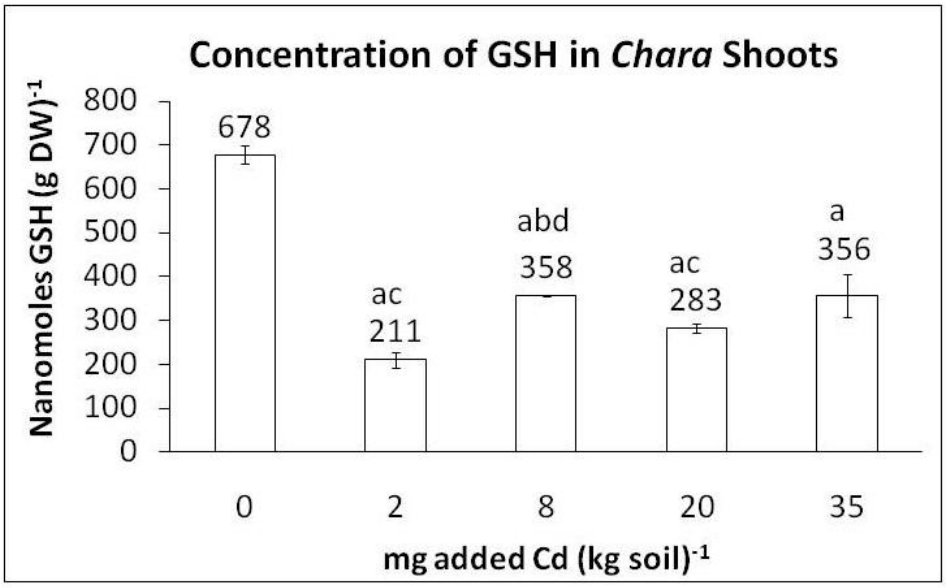


**FIGURE S1. Concentration of glutathione in *Chara australis* shoots in response to Cd exposure.** Explants were cultured in soil containing up to 35 added mg Cd (kg soil)$^{-1}$ for 97 days, and levels of glutathione (GSH) were measured. Rhizoid biomass was not sufficient for analysis. Data are represented as means ± SE of three replicate homogenates from 1 to 2 explants. Different letters indicate significant differences from shoots cultured in (a) 0 mg added Cd (kg soil)$^{-1}$ and (b) 2 mg added Cd (kg soil)$^{-1}$ (c) 8 mg added Cd (kg soil)$^{-1}$ or (d) 20 added mg Cd (kg soil)$^{-1}$. Statistics are based on one way ANOVA followed by Games-Howell post hoc comparisons ($p<0.05$).