



# Metal bioremediation by CrMTP4 over-expressing *Chlamydomonas reinhardtii* in comparison to natural wastewater-tolerant microalgae strains

DOI:

[10.1016/j.algal.2017.03.002](https://doi.org/10.1016/j.algal.2017.03.002)

## Document Version

Accepted author manuscript

[Link to publication record in Manchester Research Explorer](#)

## Citation for published version (APA):

Ibuot, A., Dean, A. P., McIntosh, O., & Pittman, J. (2017). Metal bioremediation by CrMTP4 over-expressing *Chlamydomonas reinhardtii* in comparison to natural wastewater-tolerant microalgae strains. *Algal Research*, 24(A), 89-96. Advance online publication. <https://doi.org/10.1016/j.algal.2017.03.002>

## Published in:

Algal Research

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1 **Metal bioremediation by *CrMTP4* over-expressing *Chlamydomonas reinhardtii* in**  
2 **comparison to natural wastewater-tolerant microalgae strains**

3  
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29 **Abstract**

30 Metal pollution in freshwater bodies is a long-standing challenge with large expense required  
31 to clean-up pollutants such as Cd. There is widespread interest in the potentially low-cost  
32 and sustainable use of biological material to perform bioremediation, such as the use of  
33 microalgae. Efficient metal bioremediation capacity requires both the ability to tolerate metal  
34 stress and metal accumulation. Here, the role of a *Chlamydomonas reinhardtii* metal  
35 tolerance protein (MTP) was examined for enhanced Cd tolerance and uptake. The *CrMTP4*  
36 gene is a member of the Mn-CDF clade of the cation diffusion facilitator family of metal  
37 transporters but is able to provide tolerance and sequestration for Mn and Cd, but not other  
38 metals, when expressed in yeast. Over-expression of *CrMTP4* in *C. reinhardtii* yielded a  
39 significant increase in tolerance to Cd toxicity and increased Cd accumulation although  
40 tolerance to Mn was not increased. In comparison, the metal tolerance of three chlorophyte  
41 microalgae strains (*Chlorella luteoviridis*, *Parachlorella hussii*, and *Parachlorella kessleri*)  
42 that had previously been adapted to wastewater growth was examined. In comparison to  
43 wild type *C. reinhardtii*, all three natural strains showed significantly increased tolerance to  
44 Cd, Cu, Al and Zn, and furthermore their Cd tolerance and uptake was greater than that of  
45 the *CrMTP4* over-expression strains. Despite *CrMTP4* gene over-expression being a  
46 successful strategy to enhance the Cd bioremediation potential of a metal-sensitive  
47 microalga, a single gene manipulation cannot compete with naturally adapted strain  
48 mechanisms that are likely to be multigenic and due in part to oxidative stress tolerance.

49

50 **Keywords:** bioremediation, cadmium uptake, metal tolerance, manganese transport,  
51 *Chlamydomonas reinhardtii*, wastewater

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## 57 **1. Introduction**

58           The potential of using plants and algae for metal bioremediation has led to  
59 widespread evaluation of natural species that have innate or adapted ability for metal  
60 tolerance and accumulation [1-3]. Furthermore, the genetic engineering of photosynthetic  
61 organisms for increased tolerance, uptake, sequestration, transport and chelation of metals  
62 has been explored [4]. Macroalgae and unicellular microalgae are particularly attractive for  
63 the bioremediation of aquatic environments and wastewaters and a number of natural strains  
64 have been identified that are tolerant to a range of metals and show high metal removal  
65 abilities [2]. The mechanisms of metal tolerance and accumulation in microalgae appear to  
66 be varied, due in part to different responses to different metals, and include metal binding to  
67 the cell wall and secreted extracellular polysaccharides, intracellular metal binding peptides  
68 and proteins such as phytochelatins, glutathione abundance, oxidative stress tolerance, and  
69 metal transporter activity [5-9]. Many of these mechanisms are understood genetically and  
70 so there is potential to genetically enhance some of these algal characteristics. However, in  
71 contrast to higher plants, so far there have been very few examples of genetic engineering of  
72 microalgae to increase metal tolerance and accumulation.

73           Expression of a chicken class II metallothionein gene in the model green microalga  
74 *Chlamydomonas reinhardtii* led to increased cell growth in the presence of 40  $\mu\text{M}$  Cd and  
75 increased Cd binding and removal efficiency relative to wild type, likely due to direct Cd  
76 chelation and sequestration by the metallothionein protein [10]. Chelation of some metals  
77 occurs via histidine binding and this has been recently exploited in *C. reinhardtii* by over-  
78 expression of the *HISN3* gene, which encodes the enzyme in the fourth step of the histidine  
79 biosynthesis pathway. The transgenic microalgae showed an approximately 50% increase in  
80 histidine concentration under Ni exposure conditions and increased tolerance to Ni stress  
81 [11]. Furthermore, the accumulation of metals including Ni, as well as Zn, Cu and Mn, was  
82 increased in the *HISN3* over-expressing *C. reinhardtii* compared to wild type. In addition to  
83 metal binding, metal tolerance can be achieved via anti-oxidant or osmotic protectant  
84 activities. Proline accumulation has been linked to the tolerance of a variety of abiotic

85 stresses, and increased accumulation of proline in transgenic *C. reinhardtii* expressing a  
86 mothbean  $\Delta$ 1-pyrroline-5-carboxylate synthetase (*P5CS*) gene mediated increased Cd  
87 tolerance, possibly due to enhanced anti-oxidant activity and induction of Cd-binding  
88 phytochelatin synthesis [12]. All of these examples of microalgal engineering have evaluated  
89 improvements to metal chelation and oxidative stress response but as yet there are no  
90 examples of metal transporter over-expression in microalgae.

91 At a cellular level, the manipulation of metal transporter proteins has the potential to  
92 enhance metal accumulation into a cell, if metal uptake transporters are over-expressed, or  
93 to increase metal tolerance and internal storage, if organelle metal uptake transporters are  
94 over-expressed. There are a number of examples in higher plants where this latter strategy  
95 has been examined, particularly for tonoplast-localised transporters that perform vacuolar  
96 metal sequestration [13-16]. One class of metal transporter involved in metal sequestration  
97 and internal metal transport are the Cation Diffusion Facilitators (CDF) that are also called  
98 Metal Tolerance Proteins (MTP) in plants and algae [7, 17]. Members of this family transport  
99 metal ions like  $Zn^{2+}$ ,  $Mn^{2+}$ ,  $Cd^{2+}$ ,  $Co^{2+}$  and  $Fe^{2+}$ , and can be phylogenetically classified within  
100 a Zn-CDF, Mn-CDF or Fe/Zn-CDF clade [18]. Many MTPs are vacuolar proteins such as  
101 ShMTP8 from *Stylosanthes hamata* that is responsible for vacuolar  $Mn^{2+}$  sequestration [14],  
102 AtMTP3 from *Arabidopsis thaliana* that can mediate  $Zn^{2+}$  and  $Co^{2+}$  sequestration [19], or  
103 CsMTP1 from *Cucumis sativus* that transports  $Zn^{2+}$  and  $Cd^{2+}$  [20]. Others are localised in the  
104 secretory pathway, such as the  $Mn^{2+}$  transporting AtMTP11 [21], or at the plasma  
105 membrane, such as the  $Mn^{2+}$  and  $Cd^{2+}$  transporting CsMTP9 [22]. In many of these studies,  
106 increased expression of an MTP gene often in yeast, led to enhanced cellular metal  
107 accumulation and tolerance to high metal concentration, indicating that MTP genes are  
108 potential targets for bioremediation studies.

109 Five MTP genes have been predicted in the *C. reinhardtii* genome [7] and one of  
110 these, *CrMTP1* was shown to be transcriptionally induced under Zn deficiency conditions  
111 [23] while *CrMTP2* and *CrMTP4* are induced by Mn deficiency [24]. However, none of the  
112 microalgae MTP genes have yet been directly functionally characterised or evaluated as a

113 target for genetic manipulation. Here we describe the cloning and characterisation of  
114 *CrMTP4* and the evaluation of *C. reinhardtii* lines over-expressing this gene with regard to  
115 Mn and Cd tolerance and transport ability. In addition, these transgenic strains were  
116 compared with natural strains of chlorophyte microalgae that had been previously obtained  
117 from a metal-containing municipal wastewater environment; *Chlorella luteoviridis* and  
118 *Parachlorella hussii* [25], or had been acclimated under laboratory conditions to tolerate  
119 wastewater; as with *Parachlorella kessleri* [26]. All three strains had been found to tolerate  
120 wastewater conditions in part due to increased oxidative stress tolerance, but the specific  
121 abilities of these strains to tolerate and accumulate metals has not been previously  
122 examined.

123

## 124 **2. Materials and methods**

### 125 *2.1. Microalgae strains and growth conditions*

126 *C. reinhardtii* wild type strain CC125 was obtained from the Chlamydomonas  
127 Resource Center. *P. kessleri* (CCAP 211/11G) was originally obtained from the UK Culture  
128 Collection of Algae and Protozoa (CCAP), Oban, Scotland, UK and was subsequently  
129 acclimated for growth in municipal secondary-treated wastewater conditions as described  
130 previously [26]. *C. luteoviridis* and *P. hussii* were previously obtained from a municipal  
131 wastewater secondary treatment pond as described previously [25]. *C. reinhardtii* strains  
132 over-expressing *CrMTP4* were generated as described below. Strains were grown photo-  
133 heterotrophically in batch culture in Tris–acetate–phosphate (TAP) medium at pH 7 [27] in  
134 200 ml glass flasks on an orbital shaker rotating at 2 Hz or in 50 ml Nunc flasks, at 25°C  
135 under cool-white fluorescent lights ( $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) with a 16-h:8-h light:dark regime. For  
136 metal tolerance and accumulation experiments strains were grown in TAP media  
137 supplemented with various concentrations of  $\text{Al}_2(\text{SO}_4)_3$ ,  $\text{CdCl}_2$ ,  $\text{CuSO}_4$ ,  $\text{MnCl}_2$ ,  $\text{ZnSO}_4$  as  
138 indicated in the Results section. All cultures were inoculated with the same starting cell  
139 density as determined by cell counting to give an initial cell count of  $\sim 65 \times 10^3 \text{ cells ml}^{-1}$ .

140

141 2.2. *MTP4* cloning and bioinformatic analysis

142 *C. reinhardtii* *MTP4* (Cre03.g160550) sequence and gene model information was  
143 obtained from Phytozome v.9.1 using v.5.3 of the *C. reinhardtii* genome annotations.  
144 Phylogenetic relationship at the amino acid level was performed using full length sequences,  
145 as described previously [28]. The genome ID numbers or accession numbers for the  
146 sequences used are: AtMTP1 (At2g46800), AtMTP3 (At3g58810), AtMTP6 (At2g47830),  
147 AtMTP7 (At1g51610), AtMTP8 (At3g58060), AtMTP11 (At2g39450), CsMTP1 (EF684941),  
148 CsMTP9 (AFJ24702), OsMTP1 (Os05g03780), OsMTP8.1 (Os03g12530), PtrMTP11.1  
149 (EF453693), ShMTP8 (AY181256), ScZRC1 (YMR243C), ScCOT1 (YOR316C), ScMSC2  
150 (YDR205W), ScMMT1 (YMR177W). RNA was isolated from exponential growing *C.*  
151 *reinhardtii* CC125 cells using Trizol reagent (Life Technologies) and further purified by  
152 phenol/chloroform extraction and precipitation with isopropanol. The full length *CrMTP4*  
153 cDNA (1,617 bp) was amplified by RT-PCR using 1 µg of DNase-treated RNA using  
154 Superscript III reverse transcriptase (Life Technologies) and an oligo(dT) primer, then KAPA  
155 HiFi DNA polymerase (Kapa Biosystems) and gene-specific primers MTP4XbaIF (5'-AAA  
156 TCT AGA ATG TCG CAA CTA ACG CGC GAA G-3'; XbaI restriction enzyme site  
157 underlined) and MTP4SaclR (5'- AAA GAG CTC TCA CAG CAG ATT GAG AGC CTC GCT  
158 G-3'; SacI restriction enzyme site underlined). Genomic DNA was isolated from *C. reinhardtii*  
159 CC125 as described previously [29]. A *CrMTP4* genomic DNA fragment spanning the exon  
160 and intron regions (3,067 bp) was amplified using KAPA HiFi DNA polymerase and the  
161 MTP4XbaIF/MTP4SaclR primers. For all PCR amplification conditions, an annealing  
162 temperature of 60 °C and 35 amplification cycles were used. Following amplification, the  
163 PCR products were cloned into pGEM-T Easy plasmid (Promega) for propagation and  
164 sequencing (GATC Biotech) to confirm sequence fidelity. *CrMTP4* cDNA was sub-cloned  
165 into the XbaI and SacI sites of the yeast expression plasmid piUGpd [30] to allow expression  
166 under control of the constitutive yeast GAPDH promoter and selection of the *URA3* gene.  
167 *CrMTP4* genomic DNA was sub-cloned into the EcoRI site of the Gateway entry plasmid  
168 pENTR1A (Life Technologies) for subsequent recombination using an LR Clonase reaction

169 (Life Technologies) into the destination plasmid pH2GW7 [31] to allow expression of  
170 *CrMTP4* in *C. reinhardtii* under control of the constitutive cauliflower mosaic virus 35S  
171 promoter and selection of the *Aph7* gene.

172

### 173 2.3. Yeast heterologous expression and metal tolerance analysis

174 Yeast (*S. cerevisiae*) strains *pmr1* (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*;  
175 *pmr1::kanMX4*) (Euroscarf, Frankfurt, Germany) and the corresponding wild type strain  
176 BY4741 (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) (Euroscarf) were each transformed using  
177 the lithium acetate-polyethylene glycol method with *CrMTP4*-*piUGpd* plasmid or empty  
178 *piUGpd* plasmid and grown at 30°C in synthetic defined medium minus uracil (SD –Ura) as  
179 described previously [32]. Expression of the *CrMTP4* cDNA in yeast was confirmed by RT-  
180 PCR using the internal *CrMTP4* primers MTP4F (5'- ACA TGT GTG TGC GGG AGT CG-3')  
181 and MTP4R (5'-CTT GTG CCG GTG CAG GGA CC-3') and RNA extracted from yeast using  
182 Trizol reagent, then RT-PCR was performed as described above. PCR products were  
183 examined on a 1% agarose gel stained with SafeView (NBS Biologicals). Metal tolerance  
184 assays were performed essentially as described previously [33] on solid SD –Ura medium  
185 with or without 3 mM MnCl<sub>2</sub> or 100 μM CdCl<sub>2</sub> metal salts, or in liquid yeast-peptone-dextrose  
186 (YPD) medium with or without 100 μM Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>, 75 μM CdCl<sub>2</sub>, 1 mM CoCl<sub>2</sub>, 1 mM CuSO<sub>4</sub>, 5  
187 mM MnCl<sub>2</sub>, or 5 mM ZnSO<sub>4</sub> metal salts. Internal Cd and Mn content in yeast grown in liquid  
188 YPD containing 30 μM CdCl<sub>2</sub> or 2 mM MnCl<sub>2</sub>, respectively, was determined by inductively  
189 coupled plasma atomic emission spectroscopy (ICP-AES) as described previously [34].

190

### 191 2.4. *C. reinhardtii* nuclear genome transformation

192 The *CrMTP4gDNA*-pH2GW7 plasmid or empty pH2GW7 plasmid were transformed  
193 into *C. reinhardtii* CC125 using biolistic bombardment as described previously [29]. Lines  
194 were selected on TAP agar medium containing 10 μg ml<sup>-1</sup> hygromycin B and further selected  
195 on fresh selection medium. Three of the lines named MTP4-OE1, MTP4-OE2 and MTP4-  
196 OE3 were studied further and were maintained in selection medium until gene expression



197 analysis and metal tolerance assays were performed. Semi-quantitative expression of  
198 *CrMTP4* was determined in the MTP4-OE strains and the control (empty pH2GW7) strain at  
199 day 3 of growth in TAP medium following cell harvest and snap-freezing in liquid N<sub>2</sub> then  
200 RNA extraction using Trizol reagent and cDNA synthesis as described above. *CrMTP4*  
201 cDNA expression was confirmed by RT-PCR using the internal MTP4F/MTP4R primers and  
202 was compared relative to expression of the constitutive control transcript *CBLP* using  
203 primers CBLPF (5'-CTT CTC GCC CAT GAC CAC-3') and CBLPR (5'-CCC ACC AGG TTG  
204 TTC TTC AG-3'). PCR amplification conditions were as described above except that 25  
205 amplification cycles were used. PCR products were examined on a 1% agarose gel stained  
206 with SafeView.

207

## 208 *2.5 Quantitative gene expression analysis*

209 *C. reinhardtii* CC125 was cultured in TAP medium then CdCl<sub>2</sub> (0.1 mM or 0.2 mM) or  
210 MnCl<sub>2</sub> (0.5 mM or 1 mM) was added to day 3 cells and left to incubate for 8 h before cells  
211 were harvested and frozen in liquid N<sub>2</sub>. RNA was isolated and cDNA was produced as  
212 described above. *CrMTP4* gene expression was determined by quantitative real-time PCR  
213 (qPCR) using the internal MTP4F/MTP4R primers, a SYBR Green core qPCR kit  
214 (Eurogentec) and a StepOnePlus machine (ThermoFisher) using the SYBR Green detection  
215 program and normalised to *CBLP* gene expression. Reactions were run in triplicate and  
216 qPCR analysis was performed as described previously [29].

217

## 218 *2.6. Microalgae analysis*

219 At regular intervals over 8 d, cultures were grown in TAP medium with or without  
220 added metals and sampled to determine cell density by cell counting using a Nexcelom  
221 Cellometer T4 (Nexcelom Biosciences) or total chlorophyll (chlorophyll a+b) concentration as  
222 described previously [26]. Metal content in microalgae after 8 d growth in metal-containing  
223 medium was performed following EDTA washing to remove external (cell wall) bound metals  
224 by ICP-AES as described previously [35]. The *in vivo* production of reactive oxygen species

225 (ROS) following 0.4 mM Cd treatment was quantified using the fluorescent stain 2',7'-  
226 dichlorofluorescein diacetate (DCFH-DA) (Sigma Aldrich) as described previously [25].  
227 Statistical analysis was performed by one-way or two-way ANOVA, as appropriate and  
228 Tukey post-hoc test using GraphPad Prism v.6.

229

### 230 **3. Results**

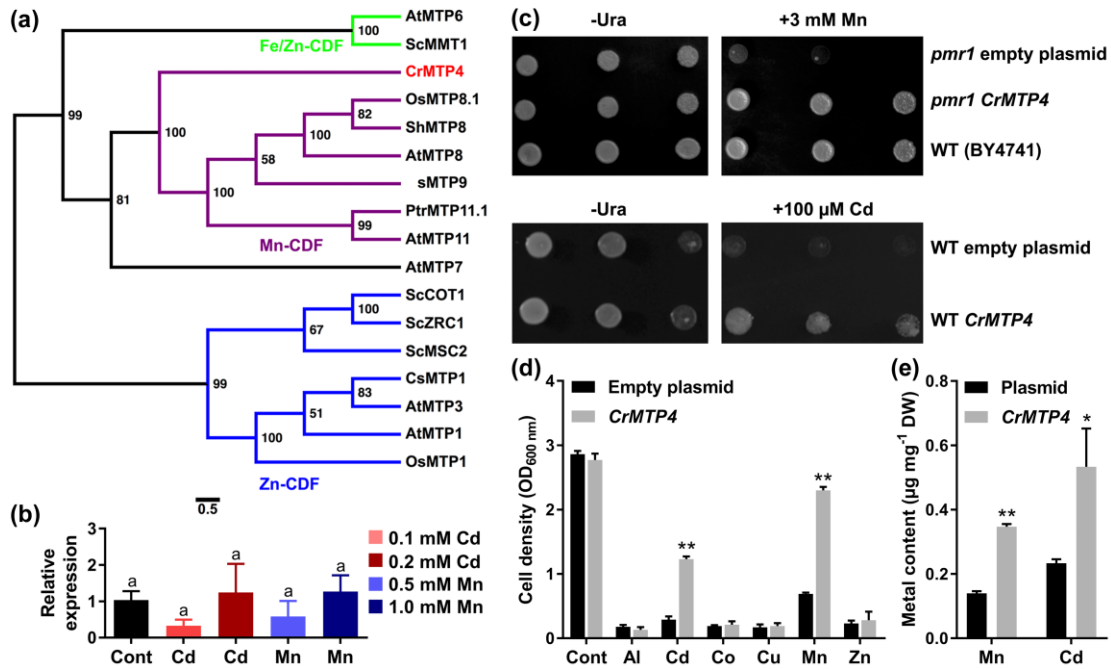
#### 231 *3.1. CrMTP4 is a Mn-CDF that can provide Mn and Cd tolerance and uptake in yeast*

232 Previous analysis of the *C. reinhardtii* genome has identified five MTP genes that are  
233 members of the CDF family [7]. *CrMTP4* is suggested to be a Mn<sup>2+</sup>-transporting CDF  
234 member but the transport specificity of this protein is unknown. Phylogenetic analysis of  
235 predicted amino acid sequence indicates that *CrMTP4* does indeed fall within the Mn-CDF  
236 clade alongside other known Mn<sup>2+</sup>-transporting MTPs including ShMTP8, AtMTP11,  
237 OsMTP8, and the Mn<sup>2+</sup>/Cd<sup>2+</sup> transporting CsMTP9, although it is distinctly clustered from the  
238 higher plant Mn-CDF proteins (Fig. 1a). To demonstrate expression of *CrMTP4* and to  
239 examine whether *CrMTP4* transcriptionally responds to Mn or Cd excess *in vivo*, day 3 *C.*  
240 *reinhardtii* CC125 cells were examined following 8 h treatment with Mn addition (up to 1 mM)  
241 or Cd addition (up to 0.2 mM); however, there was no significant difference in *CrMTP4*  
242 transcript abundance between treatments (Fig. 1b) demonstrating that *CrMTP4* expression  
243 is not transcriptionally regulated by Cd or excess Mn status, for the concentrations tested.

244 The full length *CrMTP4* cDNA was obtained by RT-PCR and metal tolerance function  
245 was confirmed by yeast heterologous expression. The cDNA sequence of *CrMTP4*, and the  
246 amino acid sequence, was identical to the predicted genome annotation. When expressed in  
247 the Mn sensitive yeast mutant strain *pmr1*, *CrMTP4* expression was able to suppress the Mn  
248 sensitivity and provide strong Mn tolerance (Fig. 1c). Following expressing in a wild type  
249 yeast strain, *CrMTP4* was able to increase tolerance to Cd (Fig. 1c and d), however, there  
250 was no change in the ability of *CrMTP4*-expressing yeast to grow in excess concentrations  
251 of other metals including Zn, Co, Cu or Al (Fig. 1d). The Mn and Cd tolerance provided by  
252 *CrMTP4* expression in yeast appeared to be due to internal sequestration as total cellular

253 concentration of Mn and Cd was significantly increased in the *CrMTP4* yeast compared to  
 254 the control, by 2.48-fold for Mn and 2.29-fold for Cd (Fig. 1e).

255



256

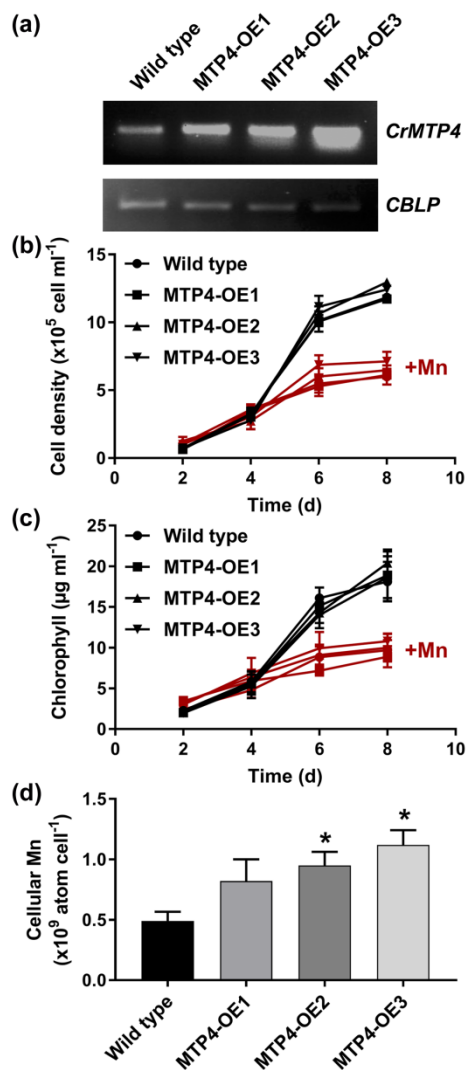
257 **Fig. 1.** Cloning of *CrMTP4* and characterization by yeast heterologous expression. (a) Phylogenetic comparison of *CrMTP4*  
 258 with selected Mn-CDF, Zn-CDF and Fe/Zn-CDF proteins from *Arabidopsis thaliana* (At), *Cucumis sativus* (Cs), *Oryza sativa*  
 259 (Os), *Populus trichocarpa* x *P. deltoides* (Ptr), *Stylosanthes hamata* (Sh) and *Saccharomyces cerevisiae* (Sc). A maximum  
 260 likelihood tree was derived from full length amino acid sequence alignment. Bootstrap values from 100 replications are  
 261 indicated and the branch length scale bar indicates the evolutionary distance of 0.5 amino acid substitution per site. (b) Gene  
 262 expression of *CrMTP4* in wild type cells in response to Cd and Mn treatment. Expression of *CrMTP4* as determined by real-time  
 263 PCR is shown relative to *CBLP* expression. Data points are means ( $\pm$ SE) calculated from 3 independent biological replicates.  
 264 Bars sharing the same lowercase letter indicate no significant difference between treatments ( $P > 0.05$ ). (c) Suppression of Mn  
 265 sensitivity of the *pmr1* yeast mutant, and Cd sensitivity of wild type (BY4741) yeast, both expressing *CrMTP4* in comparison  
 266 with empty plasmid-containing strains. Liquid cultures of strains were serially diluted then spotted onto SD -Ura medium with or  
 267 without added Mn or Cd. Yeast growth is shown after 3 d. A representative experiment is shown. (d) BY4741 strains  
 268 transformed with empty plasmid or *CrMTP4* normalized to an identical starting cell density then grown in liquid YPD medium  
 269 with or without added metals (0.1 mM Al, 75 μM Cd, 1 mM Co, 1 mM Cu, 5 mM Mn, 5 mM Zn) and grown for 24 h. Cell density  
 270 was determined by optical density measurement at 600 nm. (e) Mn and Cd uptake in EDTA-washed BY4741 yeast transformed  
 271 with empty plasmid or *CrMTP4* following growth in 2 mM Mn or 30 μM Cd YPD media. Data points are means ( $\pm$ SE) of 3  
 272 independent biological replicates. Bars indicated by asterisks show significant difference between empty plasmid and *CrMTP4*  
 273 strain within each metal treatment (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

274

275

276 3.2. Over-expression of *CrMTP4* in *C. reinhardtii* enhances Cd tolerance

277 A genomic construct of *CrMTP4* containing all predicted exons and introns was  
278 transformed into the nuclear genome of wild type *C. reinhardtii* CC125 and three lines  
279 showing strong hygromycin tolerance and significantly higher expression of *CrMTP4* relative  
280 to wild type level of *CrMTP4* expression (Fig. 2a) were chosen for further analysis. There  
281 was no significant difference in cell density or chlorophyll concentration in any of the  
282 *CrMTP4* over-expression lines compared to wild type under standard growth conditions (Fig.  
283 2b and c). Furthermore, while Mn addition to the medium up to 2 mM inhibited cell growth  
284 (Fig. 2b) and chlorophyll concentration (Fig. 2c) there was no increase in tolerance to Mn by  
285 any of the *CrMTP4* lines. However, there was evidence of a subtle but significant increase in  
286 Mn accumulation in two of the three *CrMTP4* lines (*CrMTP4-OE2* and *CrMTP4-OE3*)  
287 compared to wild type, by 1.9- and 2.3-fold, respectively (Fig. 2d).



288

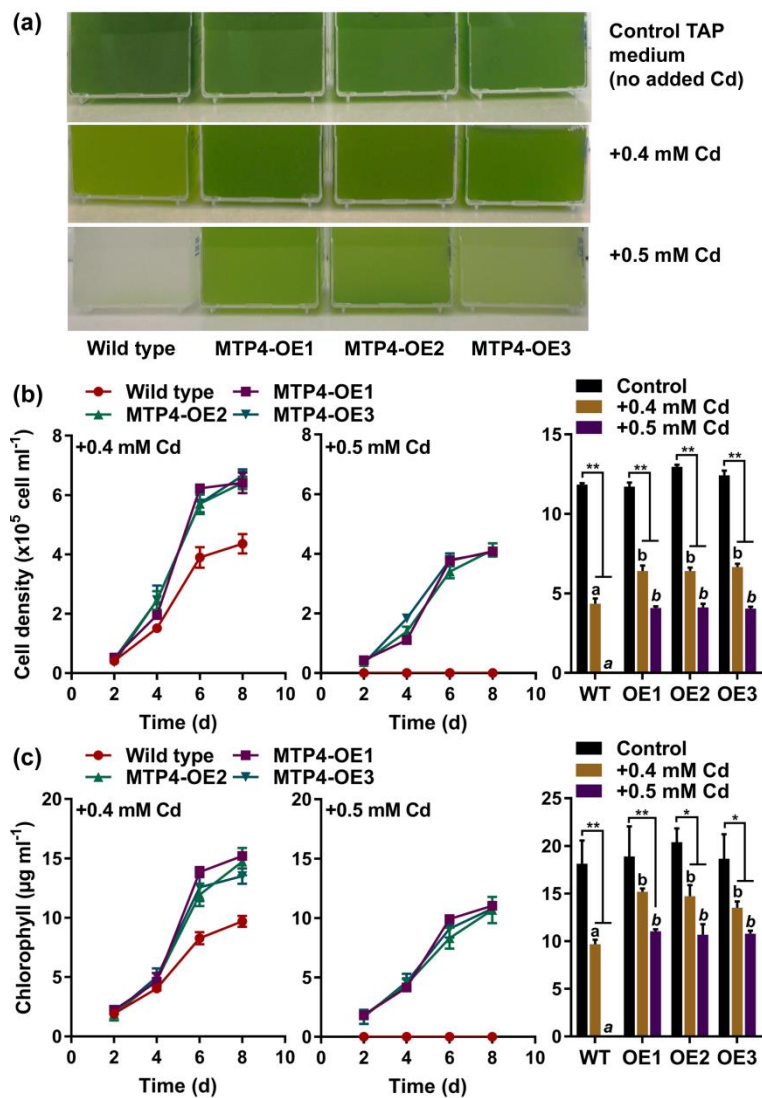
289 **Fig. 2.** Generation of transgenic *CrMTP4* over-expression lines with enhanced Mn accumulation. (a) RT-PCR detection of  
 290 *CrMTP4* mRNA transcript abundance in wild type cells in comparison to three independent *CrMTP4* overexpression (MTP4-  
 291 OE) lines. RT-PCR was performed using *CBLP* as a normalization control transcript. (b and c) Cell density as determined by  
 292 cell count measurement (b) and total chlorophyll yield (c) in wild type and MTP-OE lines over time in TAP medium with (red  
 293 symbols) or without (black symbols) 2 mM Mn addition. (d) Cellular Mn content in EDTA-washed wild type and MTP-OE cells  
 294 following growth in 2 mM Mn medium after 8 d. Data points are means ( $\pm$ SE) of 3 independent biological replicates. Bars  
 295 indicated by asterisks show significant difference between wild type and MTP-OE line (\*,  $P < 0.05$ ).

296

297 In contrast, all three *CrMTP4* lines exhibited significant tolerance to Cd addition (Fig.  
 298 3a). While the wild type cells displayed a marked loss in cell density and chlorosis in all Cd  
 299 treatments, growth and total chlorophyll yield of the *CrMTP4* lines was significantly  
 300 enhanced but there was no significant difference between the individual *CrMTP4* lines (Fig.  
 301 3b and c). In 0.5 mM Cd conditions, the wild type strain was unable to grow while *CrMTP4*

302 over-expression strains grew strongly (Fig. 3b), although growth was still significantly  
303 inhibited compared to treatments without Cd addition (Fig. 3b). To examine whether the  
304 increased stress of the strains in response to Cd addition was associated with oxidative  
305 stress, the reporter DCFH-DA for intracellular ROS production was used. *C. reinhardtii*  
306 strains were grown in TAP medium with 0.4 mM Cd addition and ROS production was  
307 measured in day 4 cells relative to cells with no added Cd. There was a  $152 \pm 26$  % increase  
308 in ROS production in wild type and a  $138 \pm 32$  % increase in the *CrMTP4* over-expression  
309 line but there was no significant difference between the strains. Growth of the *CrMTP4* over-  
310 expression lines was compared on other metal treatment conditions including Zn, Cu and Al  
311 toxicity but there was no difference in growth compared to wild type, indicating that the  
312 *CrMTP4* over-expression metal tolerance trait is specific for Cd.

313



314

315 **Fig. 3.** Cd tolerance of *CrMTP4* over-expression lines. (a) Culture phenotypes of wild type *C. reinhardtii*  
 316 overexpression (MTP4-OE) lines after 7 d growth in medium with or without Cd. A representative experiment is shown. (b and  
 317 c) Cell density as determined by cell count measurement (b) and total chlorophyll yield (c) in wild type and MTP-OE lines over  
 318 time in TAP medium with 0.4 or 0.5 mM Cd addition. Cell density and chlorophyll parameters are shown in comparison to  
 319 control treatment without Cd addition after 8 d growth (right-hand panels). Data points are means ( $\pm$ SE) of 3 independent  
 320 biological replicates. Bars indicated by different lower case letters show significant difference ( $P < 0.05$ ) within Cd treatments  
 321 and between wild type (WT) and MTP-OE lines. Bars indicated by asterisks show significant difference between control and Cd  
 322 treatments (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

323

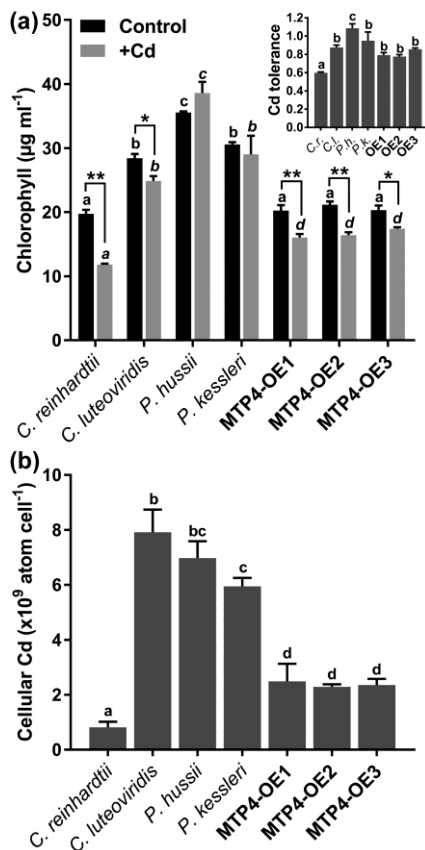
### 324 3.3. Natural wastewater adapted microalgae strains provide more substantial Cd tolerance 325 and accumulation than *CrMTP4 C. reinhardtii*

326 Microalgae that are found in polluted environments such as in wastewater are  
 327 adapted over many generations to the pollution conditions and therefore might be expected

328 to perform better with regard to metal tolerance and bioremediation than non-adapted  
329 strains. However, it is unclear how a transgenic line such the *CrMTP4* overexpression strain  
330 would compare against an adapted strain. Three microalgae strains of species *C.*  
331 *luteoviridis*, *P. hussii* and *P. kessleri*, were previously isolated from, or artificially acclimated  
332 to, municipal wastewater conditions [25, 26]. This secondary-treated wastewater was the  
333 outflow from an activated sludge treatment at a facility receiving mainly domestic wastewater  
334 and pre-treated effluent from a nearby oil refinery. The primary sedimentation and activated  
335 sludge treatment had reduced the concentration of suspended solids, biochemical oxygen  
336 demand/chemical oxygen demand, and ammonium and phosphate, but at this stage the  
337 wastewater conditions still included exposure to high concentrations of ammonium (22.7 –  
338 30.1 mg L<sup>-1</sup>) and phosphate (1.4 – 1.7 mg L<sup>-1</sup>), and bacterial contamination. Trace metals  
339 were present in the wastewater although their concentrations were fairly low (0.9 µM Cd; 2.7  
340 µM Cu; 125.2 µM Fe; 16.7 µM Mn; 18.8 µM Zn). Nevertheless, the three strains showed  
341 substantial tolerance to multiple metals compared to wild type and non-adapted *C.*  
342 *reinhardtii*. The tolerance to 150 µM Cd addition, as determined by chlorophyll content, for all  
343 three natural strains, especially *P. hussii*, was significantly higher compared to wild type *C.*  
344 *reinhardtii* but also significantly higher compared to the *CrMTP4* over-expression strains  
345 (Fig. 4a). However, it was seen that even under non-stressed (no added metal) conditions,  
346 the three wastewater-adapted strains grew better than the *C. reinhardtii* strains on the basis  
347 of total chlorophyll content. Normalized to non-stressed treatments, all three of the  
348 wastewater adapted strains still showed significantly greater tolerance than the wild type *C.*  
349 *reinhardtii*, while *P. hussii* showed significantly greater tolerance compared to the *CrMTP4*  
350 over-expression strains (Fig. 4a inset). *CrMTP4* over-expression lines were able to  
351 accumulate significantly more Cd than wild type (2.81- to 3.06-fold higher) but Cd  
352 accumulation was also significantly higher for the wastewater-adapted strains compared to  
353 both wild type and *CrMTP4* over-expressing *C. reinhardtii*; *C. luteoviridis*, *P. hussii* and *P.*  
354 *kessleri* displayed 9.7-fold, 8.6-fold, and 7.3-fold higher accumulation compared to wild type  
355 *C. reinhardtii*, respectively (Fig. 4b). For this analysis, Cd concentration was determined in



356 EDTA-washed cells in order to remove cell wall-bound metal and therefore determine  
 357 internalised metal uptake.

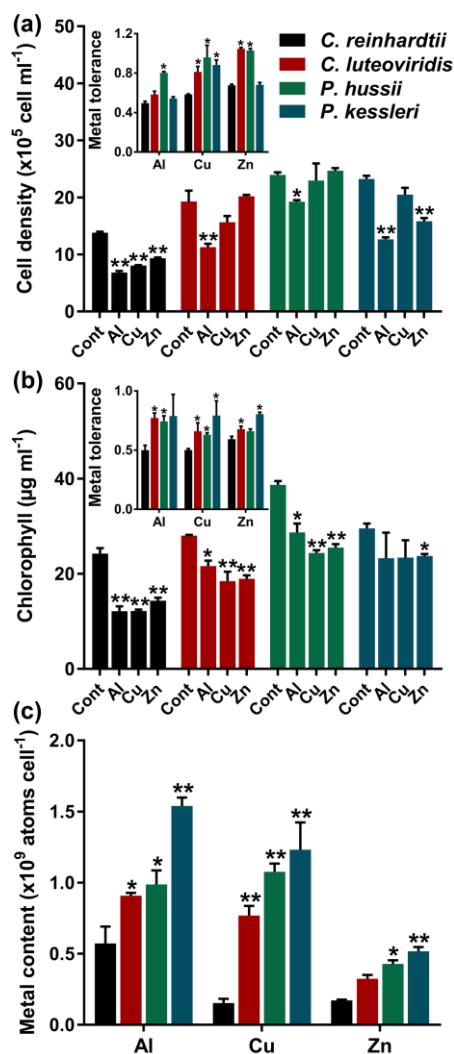


358  
 359 **Fig. 4.** Cd tolerance and accumulation of *CrMTP4* over-expression lines in comparison to natural wastewater adapted  
 360 microalgae strains. (a) Total chlorophyll yield of strains after 8 d of growth in TAP medium with or without (control) 150 µM Cd  
 361 addition. Inset: normalized chlorophyll yield of strains following Cd treatment relative to treatment without Cd. (b) Cellular Cd  
 362 content in EDTA-washed cells following growth in 150 µM Cd medium after 8 d. Data points are means (±SE) of 3 independent  
 363 biological replicates. Bars indicated by different lower case letters show significant difference ( $P < 0.05$ ) within treatments, and  
 364 pairs of bars indicated by asterisks show significant difference between control and Cd treatment (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

365  
 366 While the *CrMTP4* over-expression strains were specifically enhanced for Cd  
 367 tolerance and accumulation, the three wastewater-adapted strains also displayed increased  
 368 tolerance to other metals including Al, Cu and Zn compared to wild type *C. reinhardtii*, in  
 369 terms of cell density (Fig. 5a) and chlorophyll content (Fig. 5b), including when normalized to  
 370 values without metal addition (Fig. 5a inset and 5b inset). For all metal conditions, on the  
 371 basis of cell density, *P. hussii* was the most tolerant microalga, while *P. kessleri* showed high  
 372 metal tolerance on the basis of chlorophyll content. The internalised accumulation of Al, Cu

373 and Zn was also significantly higher for all three strains, with the exception of Zn  
 374 accumulation by *C. luteoviridis*, compared to *C. reinhardtii*, and with *P. kessleri* having the  
 375 highest accumulation of all three metals (Fig. 5c).

376



377

378 **Fig. 5.** Metal tolerance and accumulation characteristics of natural wastewater adapted microalgae strains. (a and b) Cell  
 379 density (a) and total chlorophyll yield (b) of natural strains treated without metal addition (Cont.) or with 100 µM Al, 100 µM Cu,  
 380 and 225 µM Zn in TAP medium for 8 d. Inset graphs: normalized cell density (a) and chlorophyll yield (b) of strains following  
 381 metal treatment relative to treatment without metals. (c) Cellular metal content in EDTA-washed cells following growth in 50 µM  
 382 Al, 50 µM Cu and 150 µM Zn medium after 8 d. Data points are means (±SE) of 3 independent biological replicates. Bars  
 383 indicated by asterisks show significant difference between metal treatment and control treatment, or between *C. reinhardtii* and  
 384 the wastewater adapted strain (\*, P < 0.05; \*\*, P < 0.01).

385

386

387

#### 388 4. Discussion

389 Mn-CDF genes have been identified and characterized from various higher plant  
390 species but have not as yet been examined in much detail from microalgae. Furthermore,  
391 there have been very few studies examining the bioremediation potential of MTP gene  
392 targets, and none using microalgae. Here we have functionally determined the substrate  
393 specificity of a Mn-CDF, CrMTP4 from *C. reinhardtii* and demonstrated the ability to enhance  
394 Cd tolerance and accumulation in this species of microalgae through over-expression of this  
395 Mn-CDF.

396 Phylogenetic analysis showed that CrMTP4 groups with other known Mn<sup>2+</sup>  
397 transporting MTP proteins, while previous experiments showing induction of *CrMTP4* mRNA  
398 transcript under Mn deficiency conditions had implicated this putative MTP as a Mn<sup>2+</sup>  
399 transporter [24]. Yeast heterologous expression clearly confirmed the ability of CrMTP4 to  
400 increase the cellular accumulation of Mn<sup>2+</sup> as well as to enhance tolerance of Mn in yeast,  
401 possibly due to vacuolar sequestration although this could not be confirmed. Furthermore,  
402 the yeast assay suggested that CrMTP4 also displayed Cd<sup>2+</sup> transport and tolerance activity  
403 but it could not provide yeast tolerance to other metals tested including Zn<sup>2+</sup>. Cd<sup>2+</sup> is a non-  
404 essential metal ion for photosynthetic organisms but is readily taken up and transported  
405 throughout cells often in competition with essential metal ions such as Zn<sup>2+</sup> [36], and indeed  
406 some Zn-CDF proteins such as OsMTP1 have been implicated in the transport of both Zn<sup>2+</sup>  
407 and Cd<sup>2+</sup> [37]. Many of the previously studied Mn-CDF proteins such as ShMTP8, AtMTP8,  
408 and AtMTP11 appear to be specific for Mn<sup>2+</sup> [14, 21, 38], but it is possible for Mn<sup>2+</sup>  
409 transporters to also be able to transport Cd<sup>2+</sup>. For example, the vacuolar Mn<sup>2+</sup> and Ca<sup>2+</sup>  
410 transporter AtCAX2 is also able to transport Cd<sup>2+</sup> [16], while the Mn-CDF transporter  
411 CsMTP9 was also shown to be able to transport Cd<sup>2+</sup> [22]. As a Mn<sup>2+</sup> and Cd<sup>2+</sup> transporter  
412 that was able to increase metal accumulation when expressed in yeast, CrMTP4 was  
413 therefore a suitable target to examine bioremediation potential by over-expression in *C.*  
414 *reinhardtii*.

415 A genomic construct of *CrMTP4* led to increased *CrMTP4* mRNA transcript  
416 abundance in *C. reinhardtii*, which had no obvious negative effects to the cells under non-  
417 stressed conditions. However, unlike in yeast, increased expression of CrMTP4 was unable  
418 to provide tolerance to excess Mn, despite the transgenic cells exhibiting a slight increase in  
419 Mn accumulation. Although CrMTP4 was able to promote Mn tolerance in a heterologous  
420 system, the Mn<sup>2+</sup> transport and homeostasis characteristics may be different *in vivo*. Allen *et*  
421 *al.* (2007) [24] demonstrated that out of all five *C. reinhardtii* MTP genes, *CrMTP4* showed  
422 the highest induction in response to Mn deficiency, perhaps indicating that this gene  
423 encodes a high affinity Mn<sup>2+</sup> transporter responsible for Mn<sup>2+</sup> delivery under situations of low  
424 Mn availability and therefore is poorly suited to conditions of high Mn stress. In fact we found  
425 that *CrMTP4* was not transcriptionally induced by excess concentrations of Mn, or indeed  
426 Cd. Alternatively, this may suggest that *CrMTP4* is not directly regulated by toxic  
427 concentrations of metals or possibly that regulation is post-transcriptional. While some  
428 higher plant MTP genes such as *CsMTP9* are transcriptionally induced by metal excess [22],  
429 others such as *AtMTP11* and *OsMTP8.1* are not significantly induced by high concentrations  
430 of Mn [21, 39], although *OsMTP8.1* protein abundance is moderately enhanced by high Mn  
431 supply. It is relevant to note that like for *CrMTP4*, the yeast Zn-CDF gene *ScZRC1* is also  
432 transcriptionally induced by metal limitation but shows low expression in response to high  
433 metal conditions, despite being an important component in Zn tolerance [40]. It was  
434 suggested that this is a proactive protective mechanism against a sudden switch from metal  
435 limited to excess conditions. Finally it is worth noting that the sub-cellular localization of  
436 CrMTP4 was not determined and the organelle or vesicle on which CrMTP4 resides may  
437 provide insufficient internal Mn storage to tolerate sequestration of high concentrations of  
438 Mn<sup>2+</sup>. Unlike higher plants and yeast, *C. reinhardtii* does not possess a large central vacuole  
439 but many smaller acidic vacuoles or acidocalcisomes [41, 42]. This potentially argues that  
440 internal metal sequestration as a bioremediation mechanism may be less useful for  
441 microalgae.

442 In contrast to the Mn stress response, the MTP4-OE lines all showed increased  
443 tolerance to increasing concentrations of Cd, up to 0.5 mM Cd, coupled with increased  
444 cellular content of Cd compared to wild type, suggesting internal sequestration of Cd rather  
445 than Cd efflux. In comparison to other previously generated transgenic Cd tolerant *C.*  
446 *reinhardtii* strains, the MTP4-OE lines displayed greater tolerance to Cd than the proline-  
447 accumulating *P5CS* lines that only showed a 1.5-fold increase in growth compared to wild  
448 type grown in 100  $\mu$ M Cd [12]. The MTP4-OE lines also had greater Cd tolerance compared  
449 to *C. reinhardtii* expressing a chicken metallothionein [10]. To date, this is the only example  
450 of an MTP gene being genetically engineered in an algal species to enhance potential metal  
451 bioremediation characteristics, and there are just two recent examples to compare of an  
452 MTP being over-expressed in higher plants. OsMTP1 was ectopically expressed in tobacco  
453 to give increased tolerance to 100  $\mu$ M Cd, by suppressing inhibition of growth, lipid  
454 peroxidation and cell death compared to wild type tobacco [43]. Furthermore, OsMTP1  
455 expression gave an approximately 2-fold increase in whole plant Cd accumulation compared  
456 to wild type, with the tolerance and accumulation trait likely due to increased vacuolar  
457 sequestration. Likewise, ectopic expression of CsMTP9 in *A. thaliana* increased plant  
458 tolerance to Cd and led to enhanced shoot Cd accumulation relative to the roots [22]. Unlike  
459 for CrMTP4, CsMTP4 over-expression also led to increased Mn tolerance, however, it is  
460 important to note that CsMTP4 localized to the plasma membrane and provides metal  
461 tolerance by cellular efflux.

462 Despite the increased Cd tolerance and accumulation provided by *CrMTP4* over-  
463 expression, the natural wastewater adapted strains all showed substantially greater  
464 tolerance and accumulation of Cd, as well as other metals including Al, Cu and Zn. Previous  
465 analysis of these natural strains demonstrated that they are able to tolerate the toxic effects  
466 of the wastewater environment because of high oxidative stress tolerance activities, which  
467 include increased ascorbate peroxidase activity and carotenoid accumulation compared to  
468 the non-adapted strains [25, 26]. Prolonged exposure to metals such as Cd induces cellular  
469 toxicity in part due to oxidative stress [44, 45]. Indeed it was also demonstrated here that

470 ROS accumulation was increased in *C. reinhardtii* strains in response to the Cd treatment,  
471 however, there was no significant difference in ROS production following *CrMTP4* over-  
472 expression, suggesting that the increased Cd tolerance provided by enhanced CrMTP4-  
473 mediated Cd transport activity was not due to reduced ROS accumulation. Although it  
474 cannot be ruled out that the wastewater-adapted *P. hussii*, *P. kessleri* and *C. luteoviridis*  
475 strains have higher abundance and activity of other metal tolerance mechanisms, such as  
476 metal transporters, compared to *C. reinhardtii*, these results suggest that oxidative stress  
477 tolerance activity is a better target in microalgae than enhanced metal sequestration alone.  
478 This also suggests that future genetic engineering strategies for microalgae metal  
479 bioremediation should additionally focus on manipulating oxidative stress tolerance.  
480 Moreover with potential concerns about the risks of genetically modified microalgae escape  
481 into the environment as well as regulatory restrictions [46], there is arguably currently greater  
482 attraction for natural strains rather than transgenic strains.

483

#### 484 **Acknowledgements**

485 This work was supported in part by The Leverhulme Trust (grant number F/00 120/BG to  
486 J.K.P.) and a BBSRC Activating Impact award. A.I. thanks the Government of Nigeria  
487 TETFUND for PhD studentship funding. O.A.M. is grateful for BBSRC DTP PhD studentship  
488 funding (grant number BB/M011208/1). We thank Paul Lythgoe (School of Earth and  
489 Environmental Sciences, University of Manchester) for ICP-AES analysis and thank  
490 Olumayowa Osundeko for providing wastewater metal concentration values.

491

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619 **Figure legends:**

620

621 **Fig. 1.** Cloning of *CrMTP4* and characterization by yeast heterologous expression. (a)

622 Phylogenetic comparison of *CrMTP4* with selected Mn-CDF, Zn-CDF and Fe/Zn-CDF

623 proteins from *Arabidopsis thaliana* (At), *Cucumis sativus* (Cs), *Oryza sativa* (Os), *Populus*

624 *trichocarpa* x *P. deltoides* (Ptr), *Stylosanthes hamata* (Sh) and *Saccharomyces cerevisiae*

625 (Sc). A maximum likelihood tree was derived from full length amino acid sequence

626 alignment. Bootstrap values from 100 replications are indicated and the branch length scale

627 bar indicates the evolutionary distance of 0.5 amino acid substitution per site. (b) Gene

628 expression of *CrMTP4* in wild type cells in response to Cd and Mn treatment. Expression of

629 *CrMTP4* as determined by real-time PCR is shown relative to *CBLP* expression. Data points

630 are means ( $\pm$ SE) calculated from 3 independent biological replicates. Bars sharing the same

631 lowercase letter indicate no significant difference between treatments ( $P > 0.05$ ). (c)

632 Suppression of Mn sensitivity of the *pmr1* yeast mutant, and Cd sensitivity of wild type

633 (BY4741) yeast, both expressing *CrMTP4* in comparison with empty plasmid-containing

634 strains. Liquid cultures of strains were serially diluted then spotted onto SD –Ura medium

635 with or without added Mn or Cd. Yeast growth is shown after 3 d. A representative

636 experiment is shown. (d) BY4741 strains transformed with empty plasmid or *CrMTP4*

637 normalized to an identical starting cell density then grown in liquid YPD medium with or

638 without added metals (0.1 mM Al, 75  $\mu$ M Cd, 1 mM Co, 1 mM Cu, 5 mM Mn, 5 mM Zn) and

639 grown for 24 h. Cell density was determined by optical density measurement at 600 nm. (e)

640 Mn and Cd uptake in EDTA-washed BY4741 yeast transformed with empty plasmid or

641 *CrMTP4* following growth in 2 mM Mn or 30  $\mu$ M Cd YPD media. Data points are means

642 ( $\pm$ SE) of 3 independent biological replicates. Bars indicated by asterisks show significant

643 difference between empty plasmid and *CrMTP4* strain within each metal treatment (\*,  $P <$

644 0.05; \*\*,  $P < 0.01$ ).

645

646 **Fig. 2.** Generation of transgenic *CrMTP4* over-expression lines with enhanced Mn  
647 accumulation. (a) RT-PCR detection of *CrMTP4* mRNA transcript abundance in wild type  
648 cells in comparison to three independent *CrMTP4* overexpression (MTP4-OE) lines. RT-  
649 PCR was performed using *CBLP* as a normalization control transcript. (b and c) Cell density  
650 as determined by cell count measurement (b) and total chlorophyll yield (c) in wild type and  
651 MTP-OE lines over time in TAP medium with (red symbols) or without (black symbols) 2 mM  
652 Mn addition. (d) Cellular Mn content in EDTA-washed wild type and MTP-OE cells following  
653 growth in 2 mM Mn medium after 8 d. Data points are means ( $\pm$ SE) of 3 independent  
654 biological replicates. Bars indicated by asterisks show significant difference between wild  
655 type and MTP-OE line (\*,  $P < 0.05$ ).

656  
657 **Fig. 3.** Cd tolerance of *CrMTP4* over-expression lines. (a) Culture phenotypes of wild type *C.*  
658 *reinhardtii* and *CrMTP4* overexpression (MTP4-OE) lines after 7 d growth in medium with or  
659 without Cd. A representative experiment is shown. (b and c) Cell density as determined by  
660 cell count measurement (b) and total chlorophyll yield (c) in wild type and MTP-OE lines over  
661 time in TAP medium with 0.4 or 0.5 mM Cd addition. Cell density and chlorophyll parameters  
662 are shown in comparison to control treatment without Cd addition after 8 d growth (right-  
663 hand panels). Data points are means ( $\pm$ SE) of 3 independent biological replicates. Bars  
664 indicated by different lower case letters show significant difference ( $P < 0.05$ ) within Cd  
665 treatments and between wild type (WT) and MTP-OE lines. Bars indicated by asterisks show  
666 significant difference between control and Cd treatments (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

667  
668 **Fig. 4.** Cd tolerance and accumulation of *CrMTP4* over-expression lines in comparison to  
669 natural wastewater adapted microalgae strains. (a) Total chlorophyll yield of strains after 8 d  
670 of growth in TAP medium with or without (control) 150  $\mu$ M Cd addition. Inset: normalized  
671 chlorophyll yield of strains following Cd treatment relative to treatment without Cd. (b)  
672 Cellular Cd content in EDTA-washed cells following growth in 150  $\mu$ M Cd medium after 8 d.  
673 Data points are means ( $\pm$ SE) of 3 independent biological replicates. Bars indicated by

674 different lower case letters show significant difference ( $P < 0.05$ ) within treatments, and pairs  
675 of bars indicated by asterisks show significant difference between control and Cd treatment  
676 (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

677

678 **Fig. 5.** Metal tolerance and accumulation characteristics of natural wastewater adapted  
679 microalgae strains. (a and b) Cell density (a) and total chlorophyll yield (b) of natural strains  
680 treated without metal addition (Cont.) or with 100  $\mu\text{M}$  Al, 100  $\mu\text{M}$  Cu, and 225  $\mu\text{M}$  Zn in TAP  
681 medium for 8 d. Inset graphs: normalized cell density (a) and chlorophyll yield (b) of strains  
682 following metal treatment relative to treatment without metals. (c) Cellular metal content in  
683 EDTA-washed cells following growth in 50  $\mu\text{M}$  Al, 50  $\mu\text{M}$  Cu and 150  $\mu\text{M}$  Zn medium after 8  
684 d. Data points are means ( $\pm\text{SE}$ ) of 3 independent biological replicates. Bars indicated by  
685 asterisks show significant difference between metal treatment and control treatment, or  
686 between *C. reinhardtii* and the wastewater adapted strain (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).