Evaluation of the use of algae for bioremediation of toxic metal pollutants

A thesis submitted to the University of Manchester for the degree of PhD in the Faculty of Life Sciences

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Faculty of Life Sciences
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Abstract

Metal pollution has been a great challenge in most industrialized countries as a result of waste generated from industrial activities being introduced into the environment. Unicellular green algae have been considered a potential biological tool for bioremediation of metal pollutants due to its metal sequestration properties. However, methods for further improving unicellular green algae metal sequestration by manipulating metal uptake and tolerance in unicellular green algae have not been studied in detail. In this study, a family of metal transport proteins named MTP1 – MTP4 from *C. reinhardtii* were screened by yeast heterologous expression for metal transport activity. MTP1 was able to strongly rescue the Zn and Co sensitivity of the *zrc1cot1* strain, MTP3 could weakly mediate Zn and Co growth, but MTP2 and MTP4 appeared to have no Zn or Co tolerance activity. MTP2, MTP3 and MTP4 but not MTP1 could strongly rescue the Mn sensitivity of the *pmr1* strain. When MTP4 was over-expressed in *C. reinhardtii* the strain showed a significant increase in Cd tolerance compared to the wild type, but no significant difference in Mn tolerance and uptake. AtHMA4 a Zn$^{2+}$ and Cd$^{2+}$ transporter from the plant *Arabidopsis thaliana*, which is a member of the Heavy Metal ATPase family, was also expressed in *C. reinhardtii*. HMA4 full length and C-terminal tail expression strains were screened for Zn and Cd tolerance and uptake. Both sets of strains showed a significant increase in Cd and Zn tolerance and uptake compared to the wild type. Metal tolerance and uptake was compared between the genetically engineered *C. reinhardtii* strains and unicellular green algal strains that are naturally adapted to metal tolerance which were *P. hussi*, *P. kessleri*, and *C. luteoviridis*. Results showed significant increase in Zn and Cd tolerance and uptake in the natural strains compared to the engineered strains. Therefore in addition to genetically engineered strains, naturally adapted strains could also be used as tools for effective metal bioremediation and pollutant treatment.
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1.0 Introduction

1.1 General Introduction

The influx of toxic metals into the environment as a result of industrial activities like mining and petroleum exploration (Wake, 2005) has become an issue of great concern. High concentration of heavy metals in the environment poses a great threat to plants, animals and humans. Due to their recalcitrant nature, and persistence in the environment, metals are classified among the most dangerous contaminants and as a result, one of the most studied contaminants (Monterio et al., 2012). Metals can concentrate in algae and other lower plants which form the first level of food chains, leading to the risk that metals might pass through a food chain and end up in large organisms potentially including humans (Hosono et al., 2011). While some of these toxic metals (e.g. Cd, Hg, Pb) are non-essential in most organisms, some metals (e.g. Zn, Cu, Mn) are essentially required in micro-scale quantities as trace nutrients in plants and animals. These essential metals are involved in a broad range of biochemical pathways and play significant roles in cellular activities like enzyme catalysis, nutrition and osmotic regulation (Hosono et al., 2011). Nevertheless, toxicity is experienced when all of these metals are present at high concentration. Due to the high use of metals for industrial activities, large amounts of metal-rich waste are disposed into the environment. As a result of this, plants and animals stand the risk of being endangered in such environments (Halim et al., 2003; Long et al., 2002).

Toxic metals cause particular risk to aquatic ecosystems through several routes which includes industrial and effluent discharge run off (Vardanyan et al., 2008). These metals accumulate in lower aquatic organisms and flow through the trophic levels of the food chain, such as to fish, ultimately getting to humans and causing significant health disorders like cancer and nervous system damage (Vanden Broek et al., 2002). Due to the vast damage high concentrations of toxic metals could cause to an aquatic ecosystem, many conventional techniques have been employed in the remediation of metal ions from
industrial waste water. These include chemical precipitation, which involves the addition of chemicals to form precipitates which sediment or settle allowing the contaminant to be removed. Another method is membrane technology involving the separation of the metallic particles using permeable membranes. Ion exchange is another conventional method used for metal removal from wastewater; this method involves ion exchange between an electrolyte solution and metal complex. Adsorption onto activated carbon is another technique which involves the use of a solid to remove soluble substances (like metal ions) from wastewater (Matheickal and Yu, 1996). Although there are advantages with these conventional techniques, they have many disadvantages. For example, chemical precipitation is ineffective especially at low concentrations of metals. Some of these techniques generate large volumes of residual semi-solid materials left from the waste which pose a great challenge in treatment. Also some of these techniques are costly, such as ion exchange, activated carbon adsorption and membrane technologies (Vardanyan et al., 2008). Therefore, the search for new cheap, sustainable and environmentally-friendly strategies for bioremediation of toxic metals is directed towards the use of biological organisms (including plants, bacteria and fungi). However, there is a lot of interest in the bioremediation potential of microorganisms such as unicellular green algae. This is due to the metal sequestering properties of these organisms and their ability to decrease the concentration of heavy metal ions in solutions (Volesky, 1987).

In this PhD study, the unicellular green algae *C. reinhardtii* was used as a model to study metal bioremediation. Studies of the *C. reinhardtii* genome have revealed metal transport protein families, including the cation diffusion facilitator (CDF) and heavy metal ATPase (HMA) families, which are the two major metal transport proteins considered in this study. These transporters play a significant role in the uptake of metal ions into the cytoplasm, trafficking of metals into cellular compartments, and detoxification of metal ions (Hanikenne et al., 2005). Many biological and chemical functions have been studied using *C. reinhardtii*, including photosynthesis and motility (Rochaix et al., 1998; Silflow & Lefebvre, 2001). Several genetic processes are easily controlled in this organism and as
such it is considered a reference organism for genetic analysis of unicellular green algae. Also, it is a powerful tool for microalgal study as its genome has been sequenced, genes can easily be transformed for over-expression into both the nuclear genome and chloroplast genome, and genes can be deleted by knocking them out to determine gene function (Remacle and Matagne, 1998; Harris, 2001).

1.1.1 The model organism

*C. reinhardtii* displays several qualities making its use as a model organism interesting and appreciable. This unicellular green alga, which belongs to the phylum Chlorophyta, has high similarity to the genomes of higher plants, but useful genomic comparisons can also be made with other model eukaryotic microorganisms such as *Saccharomyces cerevisiae*. *C. reinhardtii* is able to thrive in simple synthetic grown medium, therefore making it easy for studies of metal bioremediation. Gene expression as influenced by metal concentration can also be easily studied by examining the expression of target genes (Merchant et al., 2007; Kropat et al., 2011). *C. reinhardtii* can thrive well without light using acetate as an organic carbon source and with light using CO$_2$. This unique quality of *C. reinhardtii* makes its use as a model organism for the studies of metal ion regulation, uptake and tolerance feasible (Blaby-Haas and Merchant, 2012).

1.2 Unicellular green algae and metal uptake

In recent years bioremediation methods have been used for cleanup of metal pollutants from wastewater (Schiewer and Volesky, 2000). Microorganisms such as unicellular green algae can play a significant role in the removal of metal ions from industrial wastewater by adsorption of metal onto the cell wall and subsequent absorption into the cytoplasm where they are sequestered in the vacuole (Mehta and Gaur, 2005). The great advantage of using microalage for the biosorption of metals is its high efficiency in reducing the concentration of metals ions to a low level that is not costly. Biosorption techniques are
mostly directed towards the treatment of industrial wastewater with low metal ion concentration (Volesky, 1990). Environmental biotechnology studies demonstrate microorganisms as efficient tools for the removal of metals from polluted wastewater, for example, the use of immobilized algal biomass for wastewater treatment. Metal biosorption processes can be grouped into those that are metabolism-dependent and non-metabolism-dependent. For example, metal sorption needs metabolic activity to facilitate the process and hence energy is expended, while other processes do not require cell metabolism and do not require energy (Veglio and Beolchini, 1997). An example of a non-metabolism dependent process is the interaction of the metal with functional groups found on the cell wall and this is founded on physical adhesion (Tsezos and Volesky, 1982; Remacle, 1990). Unicellular green algae can use similar mechanisms to other microbes to sequester metal ions and can also synthesize phytochelatins in response to metal stress (Mehta and Gaur, 2005). The fact that unicellular green algae have an efficient energy source (via sunlight and photosynthesis) means that metal uptake can cost-effectively be scaled up. Metabolically active unicellular green algae can therefore be cultured in an open pond or bioreactor to provide a dependable supply of unicellular green algae biomass for removing metal ions from industrial wastewater (Blaby-Haas and Merchant, 2012). Nevertheless, low temperature might pose a threat to this up-scaling as it may not favour the proliferation of unicellular green algae.

Metal biosorption is a complex mechanism which is dependent on several factors including the chemical characteristics of the metal solution, environmental conditions (like temperature and pH), and biomass type, relating to whether unicellular, multicellular, cell size and cell wall characteristics (Nilanjana et al., 2008). The mechanism of metal sorption involves two process; adsorption and absorption. In adsorption, metal ions bind to the cell surface, enabled by the interaction between metals and the functional groups found on the cell surface. This is followed by absorption in which metal ions gain entrance to the cell membrane and the cytoplasm.
1.3 Metal toxicity resistance mechanisms by algal cells

High concentrations of metals can be toxic to unicellular green algae (Table 1.1). Unicellular green algae exposed to toxic metals experience oxidative stress and this causes damage to cellular components and alterations in ionic homeostasis (Yadav, 2010). However, unicellular green algae have developed various resistance mechanisms against toxic metals; some of these mechanisms employed to resist metal toxicity include synthesis of phytochelatins and metallothioneins, which play a major role in the chelation and sequestration of metal ions, hence shielding its toxicity to the cells (Hu et al., 2001). Cells could also resist toxicity of metal ions by flushing the metal ions into the surrounding environment with the help of efflux pumps, thereby helping to reduce the level of metal ions in the cell. Furthermore, metabolites could form complexes which coat the toxic metal ions and therefore prevent its toxicity (Monteiro et al., 2012; Mehta and Gaur, 2005). A change in oxidative state of metal ions can lead to the conversion from toxic to less toxic forms of ions by enzymatic processes (Monteiro et al., 2012). Of these resistance mechanisms exhibited by unicellular green algae when exposed to metal ions, the most prevalent are forming complexes with metabolites and precipitation (Mehta and Gaur, 2005; Monteiro et al., 2012). It is also worth knowing that different mechanisms predominate depending on the unicellular green algae species and the environmental conditions that induce metal stress (Mehta and Gaur, 2005; Franklin et al., 2002).
### Table 1.1: Selected metal toxicity in *C. reinhardtii*

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentrations (µM)</th>
<th>Effect of toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium</td>
<td>100 - 300</td>
<td>Growth reduction, Reduction in photosynthesis, Inhibition of nitrate assimilation, Inhibition photoactivation, Stimulation of photosynthesis</td>
</tr>
<tr>
<td></td>
<td>10 - 100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 - 50</td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>80 - 300</td>
<td>Growth reduction, Reduction of chlorophyll content, Inhibition of nitrate assimilation, Reduction of photosynthesis, Inhibition of external carbonic anhydrase</td>
</tr>
<tr>
<td></td>
<td>2 - 30</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>100 - 300</td>
<td>Inhibition of nitrate assimilation, Reduction of photosynthesis, Inhibition of carbonic anhydrase, Stimulation of photosynthesis</td>
</tr>
<tr>
<td></td>
<td>20 - 30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 - 30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 - 20</td>
<td></td>
</tr>
<tr>
<td>Aluminium</td>
<td>50 - 200</td>
<td>Reduction of photosynthesis, Changes in organelles structure (mitochondria and nucleus).</td>
</tr>
<tr>
<td></td>
<td>10 - 40</td>
<td></td>
</tr>
<tr>
<td>Cobalt</td>
<td>10 - 40</td>
<td>Reduced growth and motility, Reduction of chlorophyll content</td>
</tr>
<tr>
<td></td>
<td>10 - 50</td>
<td></td>
</tr>
</tbody>
</table>

[Adapted from Hanikenne et al., 2009 (Sources: Kobayashi et al., 2006; Vega et al., 2006; Faller et al., 2005; Devriese et al., 2001; Mosulen et al., 2003; Bowsell et al., 2002; Luis et al., 2006)]
1.4 Role of the algal cell wall in metal binding

The cell wall is the first line of defence against metal toxicity to unicellular green algae. The microalgal cell wall has been reported to have high metal binding capacity and a significant role in effectively shielding or preventing the cell from excessive metal uptake (Sandau et al., 1996; Mehta and Gaur, 2005). The cell wall is composed of various proteins and polysaccharides and these therefore increase the number of binding sites for metal ions. The variations in the quantity and type of metal ions that bind to the cell could be influenced by the composition of the cell wall and the type of organism (Nilanjana et al., 2008). The cell walls of algae contain heteropolysaccharides, for example alginate; this therefore reflects on the functional groups present which are carboxylates, sulphates, phosphates, sulfhydryls and imidazoles (Crist et al., 1981). The presence of heteropolysaccharides in green unicellular algae provides carboxyl and the sulphate groups for binding of metal ions (Lee, 1980). Green algae cell walls also contain proteins with different percentage composition of amino acids (Siegel and Siegel, 1973; Chapman, 1980; Lee, 1980; Guing and Blunden, 1991). These amino acids for example proline and histidine could play a significant role in metal ion binding and hence enhance metal tolerance (Sharma and Dietz, 2006).

1.5 The plasma membrane and metal flow

The plasma membrane separates the interior of the cell from the outside environment, thus constituting a barrier for metal flow from the outer environment into the cell cytoplasm. Many metal transport proteins are located in the plasma membrane and play a significant role in the transport of metal ions in and out of the cell (Blaby-Haas and Merchant, 2012). Recently though, a variety of biochemical, bioinformatics and functional genomics studies have been used in investigating the mechanisms involved in metal ion regulation and transport in *C. reinhardtii*. The successful completion of the sequencing of the *C. reinhardtii* genome has brought a great breakthrough in identifying the molecular mechanisms for metal transport, thus the metal transporter genes could be compared with
the ones present in other organisms. In the *C. reinhardtii* genome, at least eleven distinctive gene families that are recognized to encode metal ion transporters are represented (Hanikenne et al., 2005; Klein et al., 2006; Merchant et al., 2006). Although many metal transporters are shared with plants, bacteria and animals, recent studies have shown that the full complement of metal ion transporter families present in *C. reinhardtii* is more similar to that of yeast (*Saccharomyces cerevisiae*) (Hanikenne et al., 2005), making yeast a good system for the heterologous characterization of metal transport proteins from *C. reinhardtii*.

Different genetic approaches have been employed to enhance metal uptake and tolerance by microalgae; one is the increased expression of metal transport genes in the organism. To improve the metal binding capacity of *C. reinhardtii*, Rajamani et al. (2007) suggested the expression of a gene encoding for a metal uptake transporter in the plasma membrane which will enhance metal uptake across the plasma membrane. In a similar approach, the expression of a metallothionein protein in the cell apoplast of *C. reinhardtii* was proposed to enhance metal uptake (Fig. 1.1). Although this is expected to bind metal ions on the cell surface, the fact that this could increase binding sites probably could enhance metal uptake. These and other genetic approaches could enhance the capacity of metal flow into the cytoplasm through the cell membrane.

In another study, binding affinity has been reported for Au, Pd, Cu, Cd, Hq, Ag and Zn by a metallothionein (Stillman, 1995). *C. reinhardtii* cells genetically engineered with metal binding proteins were found to grow well unlike the wild type when exposed to high concentration of Cd, and metal uptake was reported to be five-fold more than the wild type (Sayre and Wagner, 2005). This clearly shows that heavy metal tolerance and accumulation can be manipulated through selective expression of transgenes in the apoplast of algae (He et al., 2006), a direction towards heavy metal bioremediation application.
Figure 1.1 Model of the plasma membrane-metallothionein (MT) fusion protein for enhanced heavy metal binding in transgenic *C. reinhardtii* (Sayre and Wagner, 2005).
1.6 The role of phytochelatins in metal tolerance in unicellular green algae

One of the mechanisms employed by unicellular green algae in response to metal toxicity is the synthesis of phytochelatins (Pinto et al., 2003; Hu et al., 2001; Fig.1.2). Phytochelatins are polymers of glutathione synthesised by a glutathione synthase enzyme and sequestered in the vacuole and sometimes in the chloroplast (Hu et al., 2001). Phytochelatins play a significant role in the sequestration and detoxification of metal ions with high specificity for metals including Cd, Zn, Cu, Pb, Au, Hg, Ag (Stillman, 1995). Algae cells have been shown to synthesize phytochelatins as a natural response mechanism when exposed to high concentrations of metals (Vatamaniuk et al., 2000). Synthesis of phytochelatins is therefore regulated by the metal ion concentration. Although the synthesis of phytochelatins helps to sequester metal ion in the cells, it is worthy of note that this does not provide absolute protection against metal toxicity (Rajamani et al., 2007). A complex process is therefore involved in metal detoxification.
Figure 1.2 Schematic representation of metal biosorption process in unicellular green algae including the cellular compartments like the vacuole and secretory pathway that help in metal homeostasis/detoxification; M represents metal ions – irrespective of the oxidation state. Metal ions bind to the cell wall and are taken up by the cell either through passive diffusion or mediated transport. =SH is the thiol group of the phytochelatins which is implicated in metal chelation.
1.7 The role of metal transport proteins in metal bioremediation

To adequately maintain metal homeostasis and to cope with the detrimental effect of excess essential and non-essential metals, plants including algae have developed a system of metal uptake, chelation, trafficking and metal storage processes. Metal transport proteins are needed to maintain metal homeostasis and therefore constitute a vital component of the homeostasis network (Hall and Williams, 2003; Clemens, 2001). Many factors play a significant role in metal transport including the chemical speciation of the metals, which could in turn be influenced by the pH of the medium. The direction of transport is largely dependent on the ion gradient across the membrane for passive transport processes and whether the transport protein is energised to allow the pumping of the ion against the concentration gradient, such as with an ATPase or a proton-coupled antiporter (Blaby-Haas and Merchant, 2012).

Metal transporters play a dual role in metal homeostasis; while some play the role of absorbing metals and hence increasing metal concentration in cell when there is a 'short-fall' in cellular metal concentration, others play the role of reducing the metal concentration in the cytoplasm by moving metals out of the cell or into intracellular compartments such as the vacuole. This involves distributing metals to the different compartment of the cell where needed. These well arranged but complex roles of metal transport clearly indicates the significant role these proteins play in maintaining a constant, well-balanced metal concentration in the cell, which is paramount for cell survival (Blaby-Haas and Merchant, 2012).

Although the transport specificities, expression characteristics and sub-cellular localization of these metal transport proteins in C. reinhardtii are poorly understood, recent studies are beginning to uncover some of this knowledge (Blaby-Haas and Merchant, 2012). In order
to engineer unicellular green algae for bioremediation purposes, it is important to understand the mechanisms of metal uptake, transport and storage in the cell and explain the intracellular distribution of some of the toxic metals.

1.8 Metal transport proteins

Several metal transporters from about 13 families or sub-families of proteins have been reported in *C. reinhardtii* (Table 1.2). Some of the metal transport families reported include Cation Diffusion Facilitator (CDF), Heavy metal-transport P-type ATPase (HMA), ZRT-, IRT-like protein (ZIP), Cation exchanger (CAX), Copper transporter (COPT and CTR), Fe transporter (FTR), and Natural resistance-associated macrophage protein (NRAMP) (Hanikenne et al., 2009, 2005; Blaby-Haas and Merchant, 2012). In this study, CDF and HMA metal transport families will be considered for over-expression and characterisation in *C. reinhardtii* for improved metal tolerance and uptake; thus it is worth discussing them in more detail here.

1.8.1 Cation diffusion facilitators (CDFs)

The CDFs are a family of metal transporters that are involved in the flow of various metals from the cytoplasm into subcellular compartments (e.g. vacuole or endoplasmic reticulum). Metal flow by this metal transport family can also be from the cytosolic to the extracellular environment. Metal specificity for this family includes, Zn$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Mn$^{2+}$, Fe$^{2+}$ (Gaither and Eide, 2001). Zn specific proteins are referred to as Zn-CDFs, Mn specific proteins as Mn-CDFs, and Fe/Zn specific proteins as Fe/Zn-CDFs (Montanini et al. 2007).

The structure and the composition of the proteins in this family are conserved between CDFs from various species, which includes the possession of six putative trans-membrane domains with N- and C-termini situated on the cytosolic side of membrane.
The C-terminal tail of some CDFs is proposed to have metal binding characteristics (Hanikenne et al., 2005). These metal transport proteins contain specific residues for cation efflux within transmembrane domain I to V, with a his-rich loop region between the TM domain IV and V conserved in Zn-CDFs which are Zn specific proteins (Gaither and Eide, 2001; Fig. 1.4).

The plant members of the CDF family have been named Metal Tolerance Proteins (MTPs) (Delhaize et al., 2003), and this name has also been applied to the *C. reinhardtii* MTPs (MTP1-5) (Hanikenne et al., 2005). In *C. reinhardtii*, five MTPs have been predicted from genome sequence analysis (Table 2).

There are a subgroup of Zn-transporting CDFs in plants (AtMTP1-AtMTP4), humans (HsZnT2-HsZnT4 and HsZnT8) and *S. cerevisiae* (Zrc1 and Cot1; Hanikenne et al., 2005). AtMTP1 and AtMTP3 are both implicated in the transport and storage of Zn in the vacuole (Kobae et al., 2004; Arrivault et al., 2006; Kramer, 2005). High expression of MTP1 has been reported in *Thlaspi caerulescens* and *Arabidopsis halleri*. These plants accumulate high concentrations of Cd and Zn, and the high expression of MTP1 partly explains their Zn tolerance phenotype (Cracium et al., 2006; Becher et al., 2004; Talke et al., 2006). A homologue of MTP1 is also found in *S. cerevisiae*, named Zrc1, which plays a role in Zn transport, storage and detoxification, and is predicted to localize at the vacuole (Gaither and Eide, 2001). Another *S. cerevisiae* protein, Cot1 is also a vacuolar transporter that plays a significant role in transport of Zn and Co (Gaither and Eide, 2001).

In plants, some members of this protein transport family play a role in Mn transport and provide tolerance to excess Mn. ShMTP1 (later renamed ShMTP8) has been implicated in the transport and storage of Mn in the vacuole of *Stylosanthes hamata* (Delhaize et al., 2003). In Arabidopsis, members of this family, for example AtMTP8-11 are also predicted to mediate Mn trafficking and a role in providing Mn tolerance (Peiter et al., 2007; Delhaize et al., 2007).
Figure 1.3 Topographical model of a CDF family member (MTP1) [H- His-rich loop; TMD- Transmembrane domain; the side with His-rich loop is cytosol]
1.8.2 Heavy metal–transport P-type ATPase (HMA)

HMAs are members of the P-type ATPase subfamily, a family of metal transport proteins which contains eight to twelve transmembrane domains. HMAs have a large cytoplasmic loop region (Fig 1.5), which includes the ATP-binding domain and phosphorylation domain. The C-terminal end is proposed to have metal binding properties which could play a significant role in regulating metal transportation (Williams et al., 2000). HMAs transport a wide range of metal ions; divalent cations including Zn$^{2+}$/Co$^{2+}$/Cd$^{2+}$/Pb$^{2+}$ and monovalent cations including Cu$^+$/Ag$^+$ (Axelsen and Palmgren 2001; Cobbett et al., 2003).

In plants, AtHMA2 and AtHMA4 are proposed to play a major role in the transportation of Zn$^{2+}$ (Hussain et al., 2004; Mills et al., 2003). In yeast, the characterization of these genes to ascertain their functions proposes that AtHMA3 is a Cd$^{2+}$, Pb$^{2+}$ and Zn$^{2+}$ transport protein (Gravot et al., 2004). In Arabidopsis halleri, HMA3 is also proposed to be involved in Zn$^{2+}$ transport (Cobbett et al., 2003) while in Thlaspi caerulescens HMA4 is proposed to play a role in the transport of Zn and Cd (Bernard et al., 2004; Becher et al., 2004). Plant HMAs have been expressed in other species for providing enhanced metal tolerance (Craciun et al., 2012; Gravot et al., 2004). For example, Arabidopsis halleri HMA4 was expressed in a non-Cd tolerant Arabidopsis lyrata for enhanced Cd uptake (Courbot et al., 2007).

Cu HMAs have been reported in plants, for example AtPAA1 and AtPAA2. The localization of AtPAA1 is proposed to be at outer membrane of the chloroplast (Shikanai, et al., 2003) whereas AtPAA2 is localized at the thylakoid membrane. Both proteins play a role in Cu delivery into the chloroplast. The difference in their Cu transport function is while AtPAA2 transports Cu to the lumen for onward delivery to plastocyanin (Abdel-Ghany et al., 2005) AtPAA1 transports Cu into the stroma (Shikanai et al., 2003).
C. reinhardtii is predicted to have four HMAs; HMA1-4 (Table 1.2; Merchant et al., 2006; Hanikenne et al., 2005). HMA2-4 have also been referred to as CTP1, CTP2 and CTP3 from C. reinhardtii. These proteins are homologues of AtPAA1 and AtPAA2, and are proposed to play a role in Cu transport and trafficking of Cu (La Fontaine et al., 2002; Merchant et al., 2006) but the localization of these proteins are yet to be confirmed (Hanikenne et al., 2005).
Figure 1.4 Topographical model of a HMA family member (C-terminal - putative metal binding domain; TMD- Transmembrane domain; the side with N-terminal is cytosol)
Table 1.2 Selected Metal Transport families in *C. reinhardtii* compared with other eukaryotes

<table>
<thead>
<tr>
<th>Organism</th>
<th>CDF</th>
<th>ZIP</th>
<th>CAX</th>
<th>HMA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. reinhardtii</em></td>
<td>5</td>
<td>14</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>12</td>
<td>17</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td><em>Cyanidioschyzon merola</em></td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>Neurospora crassa</em></td>
<td>9</td>
<td>7</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td><em>S. cerevisae</em></td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>Oryza sativa</em></td>
<td>10</td>
<td>12</td>
<td>6</td>
<td>9</td>
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<tr>
<td><em>Homo sapiens</em></td>
<td>9</td>
<td>14</td>
<td>-</td>
<td>2</td>
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</tbody>
</table>

[Adapted from Hanikenne et al., 2009; References; Kiranmayi and Mohan (2006); Hanikenne et al., (2005); Merchant et al., (2006); Klein et al., (2006); Koike et al., (2004)]
1.9 Mechanism of transport of common toxic metals

Some of the metals studied in this research including Zn\(^{2+}\), Cd\(^{2+}\) and Mn\(^{2+}\) are proposed to be transported by several transport protein apart from the ones discussed earlier. It is worth discussing these metals and the intracellular circulation of these metals and the different transport proteins implicated in their trafficking.

1.9.1 Zinc

One of the most abundant trace metal nutrients with significant roles in all living system is Zn. Zn plays a significant role in the functioning of several enzymes; acting as a structural and regulatory cofactor of several enzymes (Ricachenevsky et al., 2013). Its industrial relevance has made this metal commonly abundant in the ecosystem as a result of Zn-rich wastewater from industrial plants being disposed into the environment.

Zn homeostasis needs a very complex network of several cellular proteins to coordinate its uptake, transport, storage and detoxification (Yang and Chu, 2011). As mentioned earlier, some members of the CDF transporter family are implicated in the tolerance and transport of Zn into the vacuole of unicellular green based on the knowledge of Zn-CDF function in other organisms (plants and yeast), as described above. Furthermore, HMAs also play a role in Zn transport, as described above. In the case of high intracellular Zn concentration HMAs are proposed to flush zinc out of the cytoplasm to help maintain Zn balance between the external and internal cell environments (Schulz et al., 2004).

Another typical metal transport protein family that is proposed to play a significant role in the transport of Zn is the ZIP (Zrt-, Irt-like proteins) family. In plants and yeast, ZIPs are important in Zn uptake into the cell. In \textit{C. reinhardtii} and other algae, members of the ZIP family have been predicted to play a major role in Zn uptake across the plasma membrane to help in intracellular zinc balance. These members are also proposed to play some ‘house keeping role’ in the cell regarding Zn balance (Blaby-Haas and Merchant,
2012) as they play a major role in the distribution of zinc within the other compartments and organelles of the cell such as endoplasmic reticulum, vacuole and the Golgi bodies, as demonstrated by studies of ZIPs from other organisms (Huang et al., 2005; MacDiarmid et al., 2000; Lee et al., 2010). ZIPs have not yet been characterised in significant detail in *C. reinhardtii* (Blaby-Haas and Merchant, 2013). Their presence and function in the cell intracellular membranes and the plasma membrane obviously indicates their relevance in the Zn homeostasis network in the cell and could be relevant for bioremediation experiments.

### 1.9.2 Manganese

Mn is an essential element for plants as it plays some very significant roles in the photosynthetic process (Pittman, 2005). Despite its role in the photosynthetic process in plants, high concentrations in the cell could cause alterations in the metabolic processes of the cell. For a well coordinated balance in Mn concentration in the cell, a Mn homeostasis network is obviously required by the cell. Several metal transport proteins are predicted to play some major roles in the transportation of Mn in and out of the cell and also within the cell organelles (Pittman, 2005; Williams and Pittman, 2010). As described above, some members of the CDF metal transport family have been propose to be involved in Mn transport (Hanikenne et al., 2005; Delhaize et al., 2003; Pittman, 2005). No studies have yet been performed to examine the roles of *C. reinhardtii* MTPs in Mn tolerance and bioremediation although in a previous study, some MTPs have been implicated in *C. reinhardtii* Mn deficiency response (Allen et al., 2007).

In some other organisms members of the NRAMP (Natural resistance-associated macrophage protein) family have been predicted to play a major role in Mn homeostasis. NRAMP1 in *Arabidopsis thaliana* is located in the plasma membrane, and during Mn shortfall this protein is expressed in abundance (Thomine et al., 2003; Lanquar et al., 2005) and could possibly plays a role in averting the cell from Mn deficiency shock. In Arabidopsis six NRAMPs have been identified and out of these, NRAMP1, NRAMP3 and
NRAMP4 have been functionally characterised in detail, with respect to Mn transport (Curie et al., 2000; Thomine et al., 2000). While AtNRAMP3 and AtNRAMP4 are involved in Mn transport and release from the vacuole (Lanquar et al., 2010) AtNRAMP1 is shown to play a role in Mn uptake across the plasma membrane into the cell (Cailliatte et al., 2010). In *C. reinhardtii* the location of NRAMP2 protein is not known but is predicted to be expressed highly during Mn deficiency (Allen et al., 2007) and maybe its presence could help in absorbing more Mn in order to help the cell withstand the stress that Mn limitation could cause. NRAMP 1 and 3 are predicted to localize at the vacuole and secretory pathway, respectively (Hanikenne et al., 2005).

Members of the NRAMP family in *S. cerevisiae* Smf1p and Smf2p are localized to the plasma membrane and are both important in Mn uptake into yeast but only under conditions of Mn deficiency. They are present in the Golgi during Mn replete conditions but are trafficked to the plasma membrane when needed to take up Mn (Jensen et al., 2003; Dürr et al., 1998; Culotta et al., 2005).

**1.9.3 Copper**

Like many of the metals, Cu is a vital element for growth and development by plants and algae as it plays a vital role in several physiological and metabolic processes (Yruela, 2005). However, high concentration of Cu could pose some detrimental effects. For an organism to maintain a coordinated flow and concentration of Cu in the cell and its environment, a well organised network of Cu transport proteins are involved. In eukaryotes, Cu transport proteins families have been identified to be COPTs and CTRs (Hanikenne et al., 2005). In *C. reinhardtii*, four Cu transport proteins have been identified; COPT1 and CTR1, CTR2, and CTR3 (Hanikenne et al., 2008.) The CTR family play a significant role in transport of high concentration of Cu (Blaby-haas and Merchant, 2012). *C. reinhardtii* CTR1 and CTR2 are proposed to be located in the plasma membrane and play a role in transport of Cu into the cell during Cu deficiency to maintain Cu homeostasis (Page et al., 2009). It is worth knowing that related metal proteins are present in *S.*
cerevisiae which are predicted to play the role in Cu trafficking (Hanikenne et al., 2005; Merchant et al., 2006).

As discussed earlier, C. reinhardtii CTP1, CTP2 and CTP3 are also putative Cu transporters in the sub-family of HMA (Page et al., 2009; Hanikenne et al., 2005; Merchant et al., 2006). These proteins are suggested to play a role in Cu delivery to the secretary pathway (La Fontaine et al., 2002) probably to help in Cu efflux out of the cytoplasm when the intracellular concentration is higher than needed by the cell.

1.9.4 Cobalt

Co is a scarce element in the earth’s crust; its concentration is about 25 µg/g (Hamilton, 1994). Co is reported to play a biological significant role and hence needed by plants and algae for enhancement of some metabolic processes (Bruland et al., 1991; Holm-Hansen et al., 1954). However, its major function in plants has not been confirmed yet (Marschner, 1995). Co–dependent enzymes are rare and several organisms have substituted this enzyme with alternative non Co-dependent enzymes. For example, all plants seemingly have lost the vitamin B₁₂-(cobalt) dependent enzymes, including most unicellular eukaryotes (Sim and Chow, 1999). If the concentration of Co exceeds the limit required by the cell, toxicity might be experienced.

1.9.5 Aluminium

Al is reported to be the third most abundant element in the earth’s crust (May and Nordstrom, 1991) and there has been no confirmed biological role of this metal (Egbuna and Bose, 2005). Al is toxic to plants at low concentration, including algae. In plants, members of the Nramp transport protein have been proposed to play a significant role in the transport of Al. Nrat1, a member of the Nramp family, plays the role of an Al transporter in rice (Xia et al., 2010). In other studies, the wheat ALMT1 gene is also reported to encode an Al-activated malate transport protein which is proposed to confer Al
resistance to the plant (Motoda et al., 2007) and it is reported to localize in the plasma membrane (Sasaki et al., 2004; Raman et al., 2005). In a similar study, Sasaki et al. (2004) and Delhaize et al. (2004) have demonstrated the replication of the same function in rice and barley when expressed with ALMT1. An Al transporter has not been confirmed in *C. reinhardtii* and no work has been done in this direction yet. Though Al has no known biological role in algae its transport could be mediated by some unknown transporters.

1.9.6 Cadmium

Cd is one of the metals with no biological significance; its presence negatively affects the cell (Benavides et al., 2005). The fact that Cd does not play any beneficial role in the cell means that its concentration in the cell needs to be checked to avoid detrimental effects. Metal transport families are therefore predicted in the transport of Cd in plants including algae. The CDF metal transport family and HMA are suggested to play a role in Cd transport (Hanikenne et al., 2008; Gaither and Eide, 2001), although the particular member of this family that mediates Cd transport is not yet known. The ZIP family members have also been proposed to transport Cd (Guerinot and Eide, 1999). No member of this family has also been particularly implicated in the transport of Cd in algae. It is obvious that since Cd does not have a significant biological in the cell, its transport could be to the vacuole where it is sequestrated and detoxified by metal chelators. Another mechanism for resisting the toxicity of this metal could be the direct efflux of the metal out of the cell. There have been few studies on the transport of Cd in algae; CrCAX1 has been implicated to possibly play a role in Cd transport by moving Cd into the vacuole (Pittman et al., 2009).
1.10 Metal uptake by natural unicellular green algae species

While a genetic engineering approach could be a useful direction towards metal bioremediation using unicellular green algae, it is worth also considering the use of natural unicellular green algae strains isolated from metal waste/sewage environments for metal bioremediation, as these strains could possess innate metal accumulation and tolerance characteristics and therefore could also be great biological tools for this task. While thousands of algal species are known, only a few of them have been explored for their metal uptake ability and successive use in wastewater treatment. Natural strains of unicellular green algae have proven to be potential tools in the bioremediation of toxic metals. A familiar example is *Chlamydomonas acidophilla* which is known to tolerate high concentrations of metals and grow well in metal rich environments (Nishikawa et al., 2003; Lessmann et al., 1999). Studies have shown that metal uptake ability in unicellular green algae differs greatly depending on the strains and the species for example Cd uptake by *Chlorella vulgaris* and *Spirulina* sp. showed different Cd uptake capacity (Sandau et al., 1996; Ofer et al., 2003) and Cu uptake by *C. vulgaris* and *Chlorella minimata* also showed difference in uptake abilities (Lau et al., 1999; Sandau et al., 1996).
1.11 Aims and objectives

This research work was aimed at examining the characteristics of metal absorption and accumulation into unicellular green algae and assessing the potential use of unicellular green algae for metal bioremediation. The aim of this research work was to look in detail at the accumulation of toxic metals including Cd, Zn, Co, Mn, Al and Cu, in unicellular green algae and develop methods to enhance metal accumulation in this organism. These metals were chosen considering their industrial relevance, their prevalence in the environment and the devastating effect they could have on the ecosystem. Establishing a cheap, environmentally friendly approach of metal bioremediation could be a great way to conserve our environment, hence developing unicellular green algae for metal bioremediation is the overall aim of this study. C reinhardtii was adopted as the reference organism considering its metal sequestering ability, sequenced genome and ease to genetically engineer.

Studies have shown that members of metal transport protein families are implicated in the and further trafficking of metals into the vacuole for detoxification (Hanikenne et al, 2005). This work was aimed at understanding the transport mechanisms of these metal transporters and then using some of these metal transporters as targets to genetically manipulate C. reinhardtii to enhance metal uptake and tolerance. Members of the CDF family were considered as no previous characterization of C. reinhardtii MTP1-4 has been done. These proteins are well characterised in organisms such as human, yeast and plants but not in unicellular green algae.

This research work also looked at the screening of Arabidopsis thaliana HMA4, a member of the HMA transport protein family, over-expressed in C. reinhardtii for Zn and Cd. The characterization of AtHMA4 in C. reinhardtii is novel as no work has previously been done on this. These proteins are well characterised in organisms such as yeast and plants but not in unicellular green algae. The screening of this HMA4 over-expression strain for Zn
and Cd tolerance and uptake was based on the fact that this transport protein has been reported to play a significant role in the transport of Zn and Cd in *Arabidopsis thaliana*.

Finally, metal tolerance and uptake ability of the genetically engineered unicellular green algae strains with selected natural unicellular green algae strains isolated from waste sites was also considered in this work. This was to increase our understanding of whether genetically engineered unicellular green algae strains could do better in metal tolerance and uptake than some selected wild type strains from waste sites, considering the fact that these wild type strains have acclimatised to metal-rich environmental conditions and should do well in metal tolerance and uptake.
2.0 Materials and methods

2.1 Materials

2.1.1 Suppliers

All chemicals were of the highest purity available and were supplied by Fisher Scientific Ltd, UK; Eppendorf UK Ltd., Cambridge, UK; GE Healthcare, Chalfont St Giles, Bucks, UK; Melford Laboratories Ltd., Ipswich, UK; Sigma-Aldrich Company Ltd., Steinheim, Germany; StarLab (UK) Ltd.; Qiagen, UK, Thermo Fisher Scientific, Waltham, USA, Agilent Technologies, CA, USA; Bioline, London, UK; New England Biolab, UK unless otherwise stated. Unless otherwise stated all solutions were made up in sterile deionised water.

2.1.2 Media used

Experiments in this thesis were performed using the following media; unicellular green algae was grown in Tris-Acetate-Phosphate (TAP) medium, *Escherichia coli* was grown in Lysogeny broth (LB) agar and in Super Optimal broth (SOB), yeast was grown in Synthetic Defined medium minus uracil (SD-U), and Yeast Peptone Dextrose (YPD) agar. One litre of standard TAP medium was prepared by adding 25ml of TAP salt solution (containing 15g l⁻¹ NH₄Cl to give a final concentration of 7.0mM, 4g l⁻¹ MgSO₄·7H₂O to give a final concentration of 0.41mM, and 2g l⁻¹ CaCl₂·2H₂O to give a final concentration of 0.34mM), 0.375ml of potassium phosphate solution pH 7.0 (made of 288g l⁻¹ K₂HPO₄ and 144g l⁻¹ KH₂PO₄ to give a final concentration of 1mM phosphate); 1 ml of Hutner trace elements mixture and 1 ml of glacial acetic acid (to final concentration of 18mM), buffered to pH 7.2 with 20mM Tris and made to 1L with de-ionized water (Harris 1989). Hunter trace elements mixture provided the following final concentrations of metals; 184 µM B (as H₃BO₃), 6.8 µM Co (as CoCl₂), 6.3 µM Cu (as CuSO₄), 17.9 µM Fe (as FeSO₄), 25.6 µM Mn (as MnCl₂), 6.2 µM Mo (as (NH₄)₆Mo₇O₂₄), 0.27mM Na (as sodium EDTA salt) and 76.5 µM Zn (as ZnSO₄).
SD-U media was prepared by adding 6.7 g of 0.67% nitrogen base w/v amino acids, 1.92 g of yeast synthetic dropout supplement without uracil, 20 g of dextrose agar and 20 g (2%) agar added for SD-U agar) the solution was made up to 1 litre with deionised water and autoclaved for use. Yeast Peptone Dextrose agar (YPD) was prepared adding 10g yeast, 20g of peptone, 20g Dextrose (Glucose) and (20g (2%) agar added for YPD agar) made up to 1 litre with deionised water, the solution autoclaved for use. LB was prepared by adding 10g of NaCl, 10g of tryptone, 5g of yeast extract,( 20g of agar added for LB agar) and made up to a final volume of 1 litre with deionised water.

2.1.3 Yeast mutants strains
Yeast (S. cerevisiae) strains used were pmr1 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; pmr1::kanMX4) (Euroscarf, Frankfurt, Germany), zrc1 cot1 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; zrc1::natMX; cot1::kanMX4) (Drager et al., 2004) (kindly provided by Ute Krämer, Ruhr-Universität Bochum, Germany) and the corresponding wild type strain BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) (Euroscarf).

2.1.4 Bacterial strains
The bacterial strain used was competent E. coli cells XL blue 1 (Stratagene Technical Services USA/Canada /Europe) for plasmid transformation.

2.1.5 Unicellular green algae
Unicellular green algae strains used in this research were C. reinhardtii wild type strain CC125 and cell wall-deficient strain cw15 (obtained from CCAP; stock numbers CCAP11/32C and CCAP11/32CW15+, respectively) and the wild type strains of unicellular green algae used for metal tolerance and uptake screening were Parachlorella hussii (Isolated from United Utilities waste water by Dr Olumayawa Osundeko) (Osundeko et al., 2013), Chlorella luteoviridis (CCAP 211/3) and Parachlorella kessleri (CCAP 211/11G).
2.1.6 Oligonucleotide primers

Primers were ordered from Eurofins, Ebersberg, Germany. The sequences are given in Table 2.1.

Table 2.1A Oligonucleotide PCR primer sequences used for full-length DNA amplification for yeast and unicellular green algae transformation

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence (5′ - 3′)</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>CrMTP1 FOR</td>
<td>AAATCTAGAAAAATGTCAGAAAGAAGCCTCTTC</td>
<td>Xbal</td>
</tr>
<tr>
<td>CrMTP1 REV</td>
<td>AAAGAGCTCCTAGACCTGTGCGTCAGCGAGCGGC</td>
<td>Sacl</td>
</tr>
<tr>
<td>CrMTP2 FOR</td>
<td>AAAGGATCCAAAAATGGCGCAATTAGCTGTAAG</td>
<td>BamHI</td>
</tr>
<tr>
<td>CrMTP2 REV</td>
<td>AAATCTAGAAAATGGCGCAATTAGCTGTAAG</td>
<td>Xbal</td>
</tr>
<tr>
<td>CrMTP3 FOR</td>
<td>AAAGGATCCAAAAATGGCGCAATTAGCTGTAAG</td>
<td>BamHI</td>
</tr>
<tr>
<td>CrMTP3 REV</td>
<td>AAATCTAGAAAATGGCGCAATTAGCTGTAAG</td>
<td>Xbal</td>
</tr>
<tr>
<td>CrMTP4 FOR</td>
<td>AAATCTAGAAATGTCAGAAAGAAGCCTCTTC</td>
<td>Xbal</td>
</tr>
<tr>
<td>CrMTP4 REV</td>
<td>AAAGAGCTCCTCATCAGACAGATTTAGCTGAGACCGGGCTCCTG</td>
<td>Sacl</td>
</tr>
<tr>
<td>CrMTP4F</td>
<td>AAATCTAGAAATGTCAGAAAGAAGCCTCTTC</td>
<td>_</td>
</tr>
<tr>
<td>CrMTP4R</td>
<td>AAAGAGCTCCTCATCAGACAGATTTAGCTGAGACCGGGCTCCTG</td>
<td>_</td>
</tr>
<tr>
<td>MTP1Hpa1F</td>
<td>AAAGTTAACATGTCAGAAAGAAGCAGCTC</td>
<td>_</td>
</tr>
<tr>
<td>MTP1Hpa1R</td>
<td>AAAGTTAACATGTCAGAAAGAAGCAGCTC</td>
<td>_</td>
</tr>
</tbody>
</table>
Table 2.1B Oligonucleotide PCR primer sequences used for gene expression screening of transformed in yeast and unicellular green algae

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence (5′ - 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTP1F</td>
<td>CGTGTGGCTTGAGCGAAGAG</td>
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<tr>
<td>MTP1R</td>
<td>GCTTGCCTGCGATGATARCA</td>
</tr>
<tr>
<td>MTP2F</td>
<td>ATGAGTGTGCAGGAGATCGCA</td>
</tr>
<tr>
<td>MTP2R</td>
<td>GGCAGTGGCTTCATCAGCTC</td>
</tr>
<tr>
<td>MTP3F</td>
<td>ATCGAGGCTCTTGACACTGT</td>
</tr>
<tr>
<td>MTP3R</td>
<td>GTAAGGCCGCTGCTCAGCGTC</td>
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<tr>
<td>MTP4F</td>
<td>ACATGTGTGCAGGAGATCG</td>
</tr>
<tr>
<td>MTP4R</td>
<td>TTTGTGCAGGAGATGGAGCC</td>
</tr>
<tr>
<td>AtHMA4_F</td>
<td>ATGTGCTGCTGCGAGGAGAA</td>
</tr>
<tr>
<td>AtHMA4_R</td>
<td>TCACCTTTTGTCCCCAATTTTTTCT</td>
</tr>
<tr>
<td>CrMTP1internalF</td>
<td>TCTTGACAGCATGACAC</td>
</tr>
<tr>
<td>CrMTP1internalR</td>
<td>CCTCAACCGAGCATGACAGA</td>
</tr>
</tbody>
</table>
2.1.7 Vectors

Vectors were obtained from suppliers or authors of papers in which they were first described as detailed in Table 2.2.

Table 2.2 Vectors used in this studies

<table>
<thead>
<tr>
<th>Name</th>
<th>Promoter</th>
<th>Selection marker</th>
<th>Purpose</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>piUGpd</td>
<td>GPD</td>
<td><em>URA3</em> gene for minus uracil</td>
<td>Yeast expression</td>
<td>Nathan et al. (1999)</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>_</td>
<td><em>ampR</em> gene for ampicillin</td>
<td>Cloning/sequencing of A-tailed PCR Products</td>
<td>Promega UK Ltd., Southampton, UK</td>
</tr>
<tr>
<td>pH2GW7</td>
<td>CaMV35S</td>
<td><em>aphIV</em> gene for hygromycin</td>
<td>Gateway™ 35S overexpression Vector</td>
<td>Karimi et al. (2002)</td>
</tr>
<tr>
<td>pENTR 1A</td>
<td>_</td>
<td><em>nptII</em> gene for kanamycin</td>
<td>Entry vector for Gateway™ cloning</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>
2.1.8 Software

The bioinformatic analyses performed and the programmes used were; phylogenetic tree using RaxmlGui1.1 and Fig Tree, protein helices prediction using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), protein sequence alignment using clustalw2 programme (http://www.ebi.ac.uk/Tools/msa/clustalw2/), sequence shading using shadebox programme(http://www.ch.embnet.org/software/BOX_form.html), statistical analysis using Microsoft Excel 2007 and Graphpad prism 6.0.

2.2 Generating and analysing DNA constructs

2.2.1 Polymerase chain reaction (PCR)

PCR was carried out to amplify the DNA of the targeted gene using the Oligonucleotide PCR primer sequences in Table 2.1 A; PCR was carried out using Phusion hot start polymerase. For a 20µl reaction, 4µl of 5x Phusion HF Buffer was added to the PCR tube, 0.4 µl of 10 mM dNTPs was added to tube, 1µl of 10mM forward and reverse primers were added, 1µl of 500 µg of the template was added, 0.2 µl of Phusion Hot Start II DNA Polymerase (2 units/µl was added and made up to 20 µl with deionised water. In some cases Biomix Red polymerase was used. For 20µl reaction, 12.5µl of biomix ready to use reagent was added to a PCR tube, 1µM each of the forward and reverse primers were added to the tube, 1µl of 500µg the template was added and made up to 25µl with deionised water). PCR was carried out using an AB-2720, 96 well thermal cycler (Applied Biosystems, UK) with the following PCR conditions:

Initial Denaturation -95°C 2min
Denaturation -95°C 20sec
Annealing -60°C 30sec
Extension -70°C 2min
Final Extension -70°C 2min
Cycles -36
The PCR products were run on 3% Tris-acetic-EDTA (TAE) agarose gel for a small PCR products between 200-300bp) and 1% TAE agarose gel for large PCR products (between 1000-3000bp) agarose gel.

2.2.2 PCR purification

The QIAquick® PCR purification kit (QIAGEN) was used to purify DNA fragments amplified from PCR reactions, as well as from other enzymatic reactions such as restriction digests. The protocols in the manufacturer’s handbook were followed without any alterations.

2.2.3 Restriction digests

The following were combined in an Eppendorf tube: 10-100 ng DNA to be cut; appropriate restriction buffer (the suitability of buffers for use in double digests was determined using manufacturers instructions) to a concentration of 1 ×; 5 U of each restriction enzyme; XbaI and SacI for CrMTP1 and CrMTP4 cloning; BamHI and XbaI for CrMTP2 and CrMTP3 cloning; from the pGEMT- Easy vector into plUGpd; the final volume was adjusted to 20 µl with dsH₂O. The reaction was incubated at the appropriate temperature 37ºC for 1-2 h and analysed by agarose gel electrophoresis.

2.2.4 Agarose gel electrophoresis

Routinely, 1% (w/v) agarose gels were used for analysis of DNA fragments. Restriction digests yielding small fragments (< 500 bp) were analysed on a 3% (w/v) agarose gel. A 50 × stock solution of Tris-acetic-EDTA (TAE) buffer (24% (w/v) Tris-HCl, 6% (v/v) acetic acid, 50 mM EDTA) was diluted to a concentration of 1 × in dsH₂O. SafeView (NBS Biologicals, Huntingdon, UK) was added to heated gels, to a final concentration of 0.01% (v/v). The DNA samples to be analysed were mixed with 5 × loading buffer (15% (w/v)
ficoll, 0.25% (w/v) bromophenol blue) before loading. In order to determine the size of DNA fragments, 5 µl Hyperladder I (Bioline) or Hyperladder IV (Bioline) were loaded into the first well. Gels were run in 1 x TAE buffer at 90 V for approximately 30 min and analysed using a Red™ UV transilluminator (Cell Biosciences, Santa Clara, CA, USA).

2.2.5 Gel cleanup

The QIAGEN agarose gel cleanup kit was used to purify DNA from bands cut from an agarose gel visualised by a UV-transilluminator. The protocols in the manufacturer’s handbook were followed strictly.

2.2.6 DNA ligation

The following were combined in a thin-walled PCR tube: ~100 ng insert DNA; ~50 ng vector DNA; ligation buffer (Promega) 2 x; 3 µl PCR product; 1 µl DNA ligase; 1 µl pGEMT-Easy or pENTR1A vector the final volume was adjusted to 20 µl in dsH₂O. The mixture was vortexed and incubated at 16ºC overnight.

2.2.7 Gateway cloning

Genes of interest (MTP4) were ligated into a Gateway entry vector (pENTR1A) through conventional cloning and then this was used in an LR Clonase™ reaction to insert into a destination vector (pH₂GW7). Using a 1.5ml microcentrifuge tube at room temperature, 1-7 µl of the Entry clone (50-150ng) was added, 1 µl of Destination vector (150ng/µl) and the volume was made up to 8 µl with TE. The LR Clonase II enzyme was thawed on ice and mix for about 2 mins. The LR clonase enzyme was mixed briefly twice (2 seconds each time) and 2 µl of the LR clonase enzyme was added to the previously made sample, the solution was vortex briefly to mix. The reaction was incubated at 25ºC for 1 hour; 1 µl of the proteinase K solution was added to each sample to terminate the reaction, vortex briefly and incubated at 37ºC for 10 minutes.
2.2.8 *Escherichia coli* transformation

Transformation was performed using XL1-Blue competent *E. coli* cells. Cells were thawed on ice then 100µl of cells were gently mixed with 1.7 µl of β-mercaptoethanol, incubated on ice for 10 min and 0.1 - 50 ng (~2 µl) of the plasmid DNA or 1 µl of the pUC18 control DNA was added. The tubes were incubated on ice for 30 min then heat–pulsed at 42°C for 45 sec. The tubes were incubated on ice for 2 min then preheated (42°C) SOC medium (0.9 ml) was added and the cells were incubated at 37°C for 1 h with shaking at 225-250 rpm. Cells were plated onto LB agar plates containing the appropriate antibiotics (kanamycin for pENTR 1A, hygromycin for pH2GW7, ampicillin for pGEM T- Easy). The plates were incubated over-night at 37°C.

2.2.9 Plasmid isolation from *E. coli*

Plasmids were isolated from *E. coli* using either a miniprep kit (for yields in the µg DNA range) or a midiprep kit (for yields in the mg DNA range).

2.2.9.1 Minipreps

Bacterial cultures were grown overnight in 5 ml LB + selective antibiotic at 37°C with shaking. Plasmids were isolated by alkaline extraction (Birnboim and Doly, 1979). The QIAprep Spin miniprep kit (QIAGEN) was used and the protocols in the manufacturer’s handbook were followed.
2.2.9.2 Midipreps

Bacterial cultures were grown overnight in 100 ml LB + selective antibiotic at 37°C with shaking. The NucelonBond® Xtra Midi kit (Macherey-Nagel EURL, Hoerdt, France) and the protocols in the manufacturer’s handbook were followed.

2.2.10 DNA sequencing

The PCR product was cleaned and 20 µl of 20-30 ng/µl DNA (in pGEM-T Easy) was sequenced with the SP6 and T7 commercial primers by GATC Biotech Ltd.

2.3 Yeast transformation and analysis

2.3.1 S. cerevisiae transformation

Yeast strains, both wild type (BY4741) and the mutant strains (zrc1 cot1 and pmr1), were cultured in 5 ml Yeast Peptone Agar (YPD) overnight by shaking at 30°C. Cells were transferred to Eppendorf tubes in 1ml aliquots and centrifuged for 30 sec at 15000g. The supernatant was removed and the pellet was resuspended in 1 ml 1x LiAcetate–Tris-EDTA buffer and centrifuged for 30 sec at 15000g, followed by another 1x LiAcetate–TE buffer. For each transformation, 240 µl 50% PEG 3500, 36% 1 M LiAc, 50 µl boiled Salmon Sperm carrier DNA, 14 µl sterile dH2O, 20 µl miniprep plasmid DNA (MTP1 - 4 in piUGpd) or the empty plasmid (piUGpd) were added to each yeast pellet. The mixture was vortexed vigorously until the pellet was completely resuspended. The cells were then incubated at 42°C for 1 h. After the incubation, the suspension was centrifuge for 30 sec at 15000g and the supernatant was removed. The pellet was washed in 1 ml sterile dH2O then resuspended in 100 µl of sterile dH2O and plated onto selection media plates (SD-U). The plates were incubated at 30°C for 2-3 d until colonies appeared.
2.3.2 Yeast metal tolerance growth assays

Yeast strains expressing MTP1 - 4 or empty plasmid alone were grown up overnight in 5 ml SD-U medium at 30°C with shaking. The OD$_{600}$ of each of the cultures was equalised to 1.0. In order to assess the sensitivity of the different yeast strains to different metal concentrations decreasing 4-fold dilutions of 10µl of yeast were spotted onto SD-U plates with various concentrations of additional Zn (1.5mM and 2.0Mm), Co (0.8mM and 1.6mM), Mn (3mM and 5mM) and no metal for the control plate. Yeast samples were diluted in SD medium. The plates were incubated at 30°C for 2-3 d. Wild type yeast expressing MTP1 - 4 or empty plasmid alone were grown in 5 ml of SD-U medium overnight at 30°C with shaking. The OD$_{600}$ of the cells were normalised to 1.0 and 10 µl inoculums of the cells were grown in universal tubes in SD-U media treated with different added concentrations of Zn (20mM and 40mM, Co (10mM and 20mM), and Mn (20mM and 40mM) for optical density measurement and Zn (5mM and 20mM), Co (10mM and 20mM), and Mn (10mM and 20mM) for yeast dry biomass weight measurement. The tubes were incubated at 30°C for 3 d and the optical densities (OD$_{600}$) of the cells were measured. Yeast for dry weight biomass were quantified by measuring dry weight. The culture was centrifuged at 800g for 5 min. The supernatant was removed and the pellet was dried at 60°C overnight. After drying the pellets were weighed on a fine balance (Sartorius).

2.3.3 Measurement of metal uptake by yeast

Yeast strains treated with different concentrations of metals in SD-U media were harvested at day 3, 10 ml of harvested cells were EDTA washed to remove external bound metals by centrifugation at 3000g for 10 min, and cells were resuspended and mixed with 10 ml volume of 1 mM EDTA for 5 min, centrifuged and washed with 15 ml volume of Milli-Q water. Cells were centrifuged at 3000g for 10 min, and pellets were oven-dried at 60°C for 24 h. The dried pellets were digested in 0.5 ml ultrapure concentrated nitric acid (67%) at 100°C for 3 h. Samples were diluted in Milli-Q water and analysed by inductively coupled plasma emission spectroscopy (ICP-AES) (Perkin-Elmer)
Optima 5300) a technique that help in the determination of metals at low concentration in a variety of samples based upon the impulsive discharge of photons from atoms and ions that have been excited in a radio frequency discharge.

2.3.4 RNA extraction from transformed yeast

Yeast RNA was extracted using Trizol reagent (Invitrogen). Five ml of 3 d old cultures were pelleted by centrifugation and 1 ml of Trizol was added. Cells were mixed by pipetting then centrifuged at 2000 g for 10 min at 4°C. This was followed by incubation at 25°C for 5 min before adding 0.2 ml of chloroform per ml of Trizol used. Cells were vortexed with chloroform and reincubated for 3 min at 25°C. The aqueous phase was aspirated from the Eppendorf tube after centrifugation at 3000 g for 15 min at 4°C. The aspirated sample was precipitated with 0.5 ml of 100% isopropanol per ml of Trizol used and incubated for 10 min at 25°C. Precipitated RNA was centrifuged at 12000 g for 15 min at 4°C. The RNA pellet was washed with 70% ethanol, vortexed and centrifuged at 7500 g for 5 min at 4°C. The supernatant was discarded and the pellet was air-dried for 10 min at room temperature. Isolated RNA was resuspended in 100 µl of distilled water and quantified using a Micro-Volume Full Spectrum Fluorospectrometer (Nano Drop 3300, Thermo Scientific, USA).

2.4 Unicellular green algae growth and transformation

2.4.1 Unicellular green algae growth conditions

All experiments were performed using heteroautotrophic batch cultures. All cell lines were inoculated in standard TAP media and grown up to a stationary phase (day 7 of culture). Cultures were grown in a Percival incubator (AR-66L, Geneva Scientific, USA) on an orbital shaker (SSL1, Stuart, Keison, UK) at 140rpm, under a 16h: 8h light dark cycle at 25°C with photon flux of approximately 150µmol m⁻² s⁻¹.
2.4.2 Unicellular green algae DNA transformation

The biolistic transformation technique was used in the transfer of DNA into unicellular green algae

2.4.2.1 Plate preparation

Fifty millilitres of unicellular green algae cells grown in TAP medium for 3 days (at exponential phase) was centrifuged at 100g for 10 min. The cells were re-suspended in 600 µl of selection media (hygromycin TAP agar). The cells were gently spread in the centre of the plate.

2.4.2.2 DNA binding and precipitation to gold particles

Fifty µl aliquot of gold particles (1 µm) was weighed. Five µl of plasmid DNA (1 µg/µl) was added and mixed with 50 µl of 2.5 M CaCl₂ and 20 µl of 0.1 M spermidine. The aliquot was centrifuged at 100g for 10 min. The supernatant was removed and the sample was washed with 250 µl absolute ethanol and centrifuged again for 5 min. The supernatant was removed and immediately before transformation the gold-DNA was re-suspended in 60 µl absolute ethanol.

2.4.2.3. Biolistics

The He pressure was set at 1300psi and the shelves of the biobalistics machine were sterilized using 70% ethanol. The plates were placed on shelf 3 (6 cm distance). Five µl of the gold particle coated DNA was used per shot on the carrier disk. The 1100 psi rupture disk was used. The vacuum pressure was allowed to reach 27-28 mm Hg. Plates were incubated in the unicellular green algae growth chamber (Sanyo) under a 16 h: 8h light dark cycle at 25°C with photon flux approximately 150 µmol m⁻² s⁻¹) for about 3 weeks until the appearance of transformed colonies. Transformed colonies on appearance were
allowed to mature for 4 to 5 d, after which they were picked and streaked on antibiotic selection plates for growth.

2.4.3 Genomic DNA extraction

Two ml of day 7 TAP media-grown culture was centrifuged at 1500g for 10 min and 500 µl of CTAB buffer [containing 2% (w/v) CTAB, 100 mM Tris-HCl (pH 8) and 2% (v/v) beta mercaptoethanol] was added. Cells were vortexed and incubated at 65°C for 1 h. After incubation, 500 µl of phenol/chloroform/isoamylalcohol (25:24:1) (Fisher Bioreagent) was added and the mixture was vortexed. The sample was centrifuged at 10000g for 5 min at room temperature. After centrifugation, the solution separated into two layers. The upper layer (aqueous phase) was aspirated and transferred into a new Eppendorf tube. Five hundred µl of isopropanol was added and mixed and the solution was incubated on ice for 30 min. After incubation, the precipitated DNA was centrifuged for 10000 g at 4°C for 20 min. The supernatant was removed and the DNA was washed with 200 µl of 70% ethanol and re-spun for 5 min. The supernatant was removed and the pellet was air dried for about 5 min and the extracted DNA was resuspended in 50 µl of distilled water and stored in -20°C until further use. Quantification of DNA was done using a Micro-Volume Full-Spectrum Flouropectrometer (Nano Drop 3300, Thermo Scientific, USA).

2.4.4 RNA extraction from transformed unicellular green algae

C. reinhardtii RNA was extracted using Trizol reagent (Invitrogen), exactly as described for yeast RNA extraction, above.

2.4.5 cDNA synthesis

One µg of total RNA was incubated with 1 µl DNase and 2 µl of 5 x RT buffer (Invitrogen) at 37°C for 30 min. To inactivate DNase, STOP DNase solution was added and incubated at 65°C for 10 min. For reverse transcription reaction, 11 µl of treated RNA was incubated
with 1 µl of 50 mM Oligo dT primer, 1 µl of 10 mM dNTP mix, 2 µl 5 x RT buffer, 1 µl of 0.1 M DTT, 1 µl of 200U / µl Superscript III (Invitrogen) with 3 µl of distilled water to make up to 20 µl final volume. The reaction mixture was incubated at 42°C for 1 h then at 70°C for 15 min.

2.5 Unicellular green algal metal tolerance assays
Metal tolerance of unicellular green algae (transformed and wild type) was examined by cell growth measurement and chlorophyll concentration quantification.

2.5.1 Unicellular green algal cell density measurement
Growth of all strains in different treatments were measured at 2 d intervals by optical density measurements at 680nm using a Jenway UV-spectrophotometer (model Genova), and by direct cell counting. One ml of algal culture was preserved in 20 µl of Lugol’s iodine for cell counting. Cells were counted using the cellometer (Nexcelom Bioscience, Lawrence, USA).

2.5.2 Specific growth rate
Strains were cultured in TAP media and allowed to grow for 7 d. Cells were harvested and normalised on the basis of optical density. The normalised cultures were inoculated into TAP media treated with increasing concentrations of Zn and Cd ranging between 300 and 400µM for CrMTP4 over-expression strains and allowed to grow for 8 d. Cell counts of strains treated with different metal concentrations were measured using the cellometer, and growth rate was determined at the exponential growth phase using the equation \( \mu = (\ln N_1 - \ln N_0) / (t_1 - t_0) \) where \( N_0 \) and \( N_1 \) are the cell count values at the early and late exponential phase respectively, and \( t_1 \) and \( t_0 \) are the days corresponding to \( N_0 \) and \( N_1 \), respectively (Osundeko et al., 2013).
2.5.3 Chlorophyll content extraction

The ethanol method of total chlorophyll (chlorophyll a + b) extraction was adopted (Lichtenthater and Wellburn, 1983). Algal cultures exposed to different concentrations of metals were harvested at days 2, 4, 6 and 8 of growth. Five ml of culture were transferred into 10 ml centrifuge tubes and centrifuged at 2300g for 5 min, then supernatant was removed without disrupting the cell pellet. One ml of 96% ethanol was added to each cell pellet and each pellet was resuspended using a Pasteur pipette then 4 ml 96% ethanol was added. Samples were vortexed thoroughly and centrifuged again at 2300g for 5 min. Absorbance of the ethanol extract was measured at 649 nm and 665 nm. The following equations were used to calculate Chl a and Chl b as µg ml⁻¹ of the extract:

Concentration of Chlorophyll a (µgml⁻¹) = (13.95 x A₆₆₅) – (6.88 x A₆₄₉)
Concentration of Chlorophyll b (µgml⁻¹) = (24.96 x A₆₄₉) – (7.32 x A₆₆₅)
Total Chlorophyll = (Chl a + b).

2.6 Measurement of metal uptake by unicellular green algae strains

Unicellular green algae strains treated with different concentrations of metals were harvested at day 7, 10 ml of harvested cells were EDTA washed to remove external bound metals by centrifugation at 3000 g for 10 min, and cells were resuspended and mixed with 10 ml volume of 1 mM EDTA for 5 min, centrifuged and washed with 15 ml volume of Milli-Q water. Cells were centrifuged at 3000 g for 10 min, and pellets were oven-dried at 60°C for 24 h. The dried pellets were digested in 0.5 ml ultrapure concentrated nitric acid (67%) at 100°C for 3 h. Samples were diluted 1 in 10 in Milli-Q water and analysed by inductively coupled plasma emission spectroscopy (ICPAES) (Perkin-Elmer Optima 5300).
2.7 Screening of natural unicellular green algal strains for metal tolerance and uptake

Natural unicellular green algal strains were screened for different toxic metal tolerance and uptake by growth in TAP medium. The following metals were added to TAP medium prior to inoculation: Zn (as ZnSO₄), Cd (as CdCl₂), Mn (as MnCl₂), Cu (as CuSO₄), Al (as Al₂(SO₄)₃). Different concentrations of metals ranging between 75µM and 150µM were added to the TAP media then 100 µl of day 7 cultures were inoculated into the metal treated media and allowed to grow for 8 d. Cell cultures were normalised on the basis of optical density. Chlorophyll and cell growth were analysed at 2 d intervals as described above. Metal uptake analysis was carried out at day 7 as described above.

2.8 Statistical analysis

Unless otherwise stated all data shown is representative of three replicates. Unless otherwise stated significance was determined at the P < 0.05 level by one, two-way ANOVA and T-test. All statistical tests were performed using Graph Pad Prism 6.
3.0 Functional characterization of *Chlamydomonas reinhardtii* MTP1-4 by yeast heterologous expression for enhanced metal tolerance and uptake

3.1 Introduction

MTPs are plant members of the CDFs metal transporter family. Their significant role in the transportation of different metal ions has been reported (Blaby-Haas and Merchant, 2012; Delhaize et al., 2003). Though members of the CDF have been characterized in bacteria, plants, yeast and human, the detailed functional characterization of any *C. reinhardtii* MTP proteins has not been previously performed and is the focus in this chapter. CDF transporters have been reported to provide significant tolerance for metals like Zn$^{2+}$, Mn$^{2+}$, Cd$^{2+}$, and Co$^{2+}$. These metal transport proteins are found virtually in all organisms covering the Eukaryote, Eubacteria and Archaea kingdoms (Nies and Silver, 1995).

Members of the CDF family are reported to localize to various membranes of intracellular compartments of the cell including the vacuolar membrane in plants and yeast. These proteins are involved in the efflux of metals from the cytoplasm into these organelles (Peiter et al., 2007; Haney et al., 2005) to provide ion homeostasis in the cell. Higher plant MTP transporters are reported as metal efflux transporters from the cytoplasm, predominantly responsible for transporting Zn$^{2+}$ and Mn$^{2+}$. Additionally, these transport proteins are implicated in the transport of Fe$^{2+}$, Cd$^{2+}$, Co$^{2+}$ and Ni$^{2+}$ (Persans et al., 2001; Grass et al., 2005; Montanini et al., 2007). The specificity of substrate is likely observed in similar transport proteins with related phylogeny and protein structure. The characterization of *Arabidopsis thaliana* MTP1 and related MTP1 orthologues in plants has so far demonstrated the main role of this transporter in Zn$^{2+}$ homeostasis and providing tolerance to high concentrations of Zn (Yuan et al., 2012; Ricachenevsky et al., 2013). Several other plant MTPs have been found to be involved in Mn$^{2+}$ transport; for example, *Stylosanthes hamata* MTP1 (later renamed ShMTP8) has been reported to play a significant role in vacuolar Mn$^{2+}$ transport as well as other ShMTP proteins (Delhaize et al., 2003). *Arabidopsis* MTP11 has also been reported to transport Mn$^{2+}$ and provide
tolerance to high concentrations of Mn as demonstrated by heterologous expression in yeast (Delhaize et al., 2007).

AtMTP1 and AtMTP3 are implicated in the transport of Zn\(^{2+}\) and majorly play a role in the storage and sequestration of Zn in the vacuole (Kobae et al., 2004; Arrivault et al., 2006; Kramer, 2005). *Saccharomyces cerevisiae* Zrc1 and Cot1 also play a role in the transport of Zn\(^{2+}\) and Co\(^{2+}\) and are predicted to be localize to the vacuolar membrane (Hanikenne et al., 2005; Moreau et al., 2002). AtMTP1 and AtMTP3 are reported to have high sequence similarity and the protein secondary structure (Kobae et al., 2004; Desbrosses-Fonrouge et al., 2005; Arrivault et al., 2006) which is the principal reason for their similarity in metal (Zn\(^{2+}\)) specificity.

There are five CDF genes in *C. reinhardtii* which are named MTP1 - 5. This research chapter looks at examining the functionality of MTP1-4 through characterizing them by yeast heterologous expression using a Zn and Co sensitive yeast mutant strain *zrc1cot1* and a Mn sensitive yeast strain *pmr1*, and by also expressing the MTP proteins into wild type yeast to assess their metal tolerance capacity. Though the function of MTP5 is not yet known (Hanikenne et al., 2005) efforts to clone this cDNA did not yield a positive result.
3.2 Results

3.2.1 Sequence analysis of *Chlamydomonas* MTP genes.

Phylogenetic analysis was performed to examine the relationship of the *C. reinhardtii* MTP proteins with those from plants and yeast. CrMPT1 along with other plants MTPs falls in the clade of Zn transporters with the yeast metal transporter ScZRC1 and ScCOT1 (Fig. 3.1) which has been reported to transport Zn$^{2+}$ and Co$^{2+}$ (Gaither and Eide, 2001). Arabidopsis Zn$^{2+}$ transporters MTP1, MTP2, MTP3 and MTP4 (Kobae et al., 2004; Desbrosses-Fonrouge et al., 2005; Kramer, 2005; Arrivault et al., 2006) also fall in the same clade. AhMTP1 clustered in the same group and is also predicted to transport Zn$^{2+}$ and Cd$^{2+}$ since it is expressed highly in the Zn/Cd hyperaccumulator *Arabidopsis halleri* (Desbrosses-Fonrouge et al., 2005; Kobae et al., 2004). In contrast, CrMTP2-4 fell in the same group with AtMTP11 which has been reported to transport Mn (Delhaize et al., 2007). CrMTP5 is in the same clade with AtMTP5 and AtMTP7, which have no confirmed function (Hanikenne et al., 2005). Other proteins in that group include OsMTP8 and AtMTP12.

Zn-CDF gene sequence analysis shows AtMTP3, OsMTP1, AtMTP1, AhMTP1 and NcMTP1 with close conservation as indicated by the black shaded residues (Fig. 3.2) while CrMTP1 had less conservation. Similarities were observed between the Zn-CDFs of plants and *S. cerevisiae* with CrMTP1. With Mn-CDF gene sequence analysis, AtMTP9, OsMTP7, AtMTP11, OsMTP4, OsMTP3, AtMTP8 and CrMTP2 had close similarities as indicated by the black colour shading (Fig. 3.3).

As mentioned in Chapter One, members of this metal transport protein family have six transmembrane domains (TMDs) (Ricachenevsky et al., 2013). However, this may not be strictly applicable to all the CDFs as the positions of the transmembrane domains can differ from each other (Chao and Fu, 2004; Kawachi et al., 2008; Grass et al., 2005), which could be a basis for their potential differences in specificity in metal tolerance and transport.
The similarity between CrMTP1 and the other Zn-CDF proteins is shown with protein helices prediction by hydropathy analysis. A histidine stretch between TMD 4 and 5 appears to be common among the Zn-CDF proteins. Hydropathy analysis found that the His stretch in CrMTP1 is much longer than in AtMTP1 and OsMTP1 (Fig 3.4). This obvious difference in the histidine stretch makes CrMTP1 unique from the rest of the CrMTPs. In contrast, for the Mn-CDFs, the CrMTP2 - 4 proteins and the AtMTP8 - 9 proteins, all lack the long histidine stretch (Fig.3.5).
Figure 3.1 Phylogenetic tree of selected plant and yeast CDFs; the tree showing that most of the genes are in the Zn, Co and Mn clades. The MTP protein sequences used in the analysis are from Arabidopsis thaliana (At), Oryza sativa (rice Os), C. reinhardtii (Cr), Arabidopsis helleri (Ah), Noccaea caerulescens (Nc), Saccharomyces cerevisiae (Sc). Sequences were aligned using ClustalW2 (available at http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the tree was generated using RaxmlGui 1.1 and visualized using Fig tree. Genes at maroon colour stripe are predicted Zn and Co transporters, genes at purple colour stripe are predicted non function genes, genes at beach colour stripe are predicted Mn transporters, and genes at green colour stripes are only Zn transporters. Numbers show the level gene similarity. Scale bar=2 amino acid substitutions per site. Bootstrap percentage values are indicated at the tree nodes branches and indicate confidence in tree node positions.
Figure 3.2 Alignment of Zn-CDFs genes from *C. reinhardtii* and other species. Protein sequence from *Arabidopsis thaliana* (At), *Oryza sativa* (rice Os), *C. reinhardtii* (Cr), *Arabidopsis helleri* (Ah), *Noccaea caerulescens* (Nc), *Saccharomyces cerevisiae* (Sc) were aligned using the clustalw2 program ([http://www.ebi.ac.uk/Tools/msa/clustalw2/](http://www.ebi.ac.uk/Tools/msa/clustalw2/)) and shading was done using Boxshade 3.21 ([http://www.ch.embnet.org/software/BOX_form.html](http://www.ch.embnet.org/software/BOX_form.html)). Red squares represent Transmembrane domains.
Figure 3.3 Alignment of Mn-CDFs genes from *C. reinhardtii* and other species. Protein sequence from *Arabidopsis thaliana* (At), *Oryza sativa* (rice Os), *C. reinhardtii* (Cr), *Arabidopsis helleri* (Ah), *Noccaea caerulescens* (Nc), *Saccharomyces cerevisiae* (Sc) were aligned using the clustalw2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and shading was done using Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Red squares represent Transmembrane domains.
Figure 3.4 Prediction of transmembrane helices in Zn-CDF MTPs. A- CrMTP1, B- AtMTP1, C- OsMTP1. The CrMTP1 sequence is taken www.phytozome.com, and AtMTP1 and OsMTP1 taken http://www.ncbi.nlm.nih.gov/protein and the prediction of transmembrane helices was done using www.cbs.dtu.dk/services/TMHMM-2.0/.
Figure 3.5 Prediction of transmembrane helices in Mn-CDF MTPs. A-C CrMTP2 - 4, D-E AtMTP8 and AtMTP9. *C. reinhardtii* sequences are taken from www.phytozome.com, and *Arabidopsis* sequences are taken from [http://www.ncbi.nlm.nih.gov/protein](http://www.ncbi.nlm.nih.gov/protein) and the prediction of transmembrane helices was done using [www.cbs.dtu.dk/services/TMHMM-2.0/](http://www.cbs.dtu.dk/services/TMHMM-2.0/).
3.2.2 *C. reinhardtii* MTP1-4 characterization by yeast heterologous expression

MTP1-4 full-length cDNAs were amplified using the RT-PCR conditions described in Chapter 2 with the sequence having the introns appropriately spliced (Representative gel picture showing initial RT-PCR amplification shown in Fig 3.6.) MTP cDNAs were ligated into pGEM-T Easy and then into a yeast vector piUGpd (Representative pictures shown in Fig 3.6), a uracil selectable marker-containing plasmid with a constitutive Gpd promoter, to generate the plasmid constructs (Fig 3.6). These plasmids were transformed into the Zn and Co sensitive mutant yeast strain *zrc1cot1* (which is sensitive to Zn and Co because it lacks the yeast vacuolar Zn$^{2+}$ and Co$^{2+}$ transporters Zrc1 and Cot1) and the Mn sensitive mutant yeast strain *pmr1* (which is sensitive to Mn because it lacks Pmr1, a yeast Mn$^{2+}$ transporter). The empty vector (piUGpd) was also transformed into these yeast strains as a negative control. Using SD-Uracil yeast growth medium, a growth spotting assay of the MTP1-4 expressing yeast strains was done on SD-U media treated with different concentrations of Zn, Co and Mn and without metals as a control (Fig 3.7a). The results showed that MTP1 was able to strongly rescue the Zn and Co sensitivity of the *zrc1cot1* strain at 1.5mM and 2.0mM Zn, and weakly rescue same strain at 0.8mM Co, while Co at 1.6mM was toxic to all the MTP-expressing strains except for the MTP1 strain (Fig 3.7c). MTP3 could weakly mediate Zn and Co growth (Figures 3.7b and c). MTP2 and MTP4 expressing strains displayed no Zn and Co growth. MTP2-4 strongly rescued the Mn sensitivity of the *pmr1* strain at Mn concentrations of 3.0 and 5.0mM. Yeast strains with the empty vector (piUGpd) were not able to grow on any of the metal concentrations. The spotting assay on SD-U without metal had equal growth for all the strains including the wild type. The characterization result indicates the metal ion transport specificity and confirmation of function of these metal transport proteins.

The expression of each MTP cDNA in each yeast strain was confirmed by RT-PCR. Short primers of MTP1-4 were designed for the experiment. Total RNA extraction from a 3 day old yeast culture was performed and the result showed expression of MTP1-4 in all of the MTP-expressing strains (Fig 3.8).
Figure 3.6 Plasmids generated for MTP1-4 yeast transformation. CrMTP1-4 was first cloned into pGEMT-Easy (3015bp), then cut, purified and cloned into piUGpd for transformation into S. cerevisiae. Gel pictures showing: 1- MTP1 full length initial RT-PCR amplification, 2- MTP1 full length in p GEM-T Easy vector (plasmid), 3- MTP1 full length RT-PCR screening in piUGpd. Representative images.
Figure 3.7a MTP1-4 characterization by yeast heterologous expression without added metal (control). Zn and Co sensitive yeast strain zrc1cot1 and Mn sensitive yeast strain pmr1 were transformed with MTP1-4 and the empty vector piUGpd. The strains were all grown for 3 days and equalised at OD 600nm to 1.0. A four-fold dilution was done and the yeast spotted on SD-Uracil media.
Figure 3.7b MTP1-4 characterization by yeast heterologous expression with low metal concentrations (1.5mM Zn, 0.8mM Co and 3.0mM Mn). Zn and Co sensitive yeast strain zrc1cot1 and Mn sensitive yeast strain pmr1 were transformed with MTP1-4 and the empty vector piUGpd. The strains were all grown for 3 days and equalize at OD 600nm to 1.0. A four-fold dilution was done and the yeast spotted on SD-Uracil media.
Figure 3.7c MTP1-4 characterization by yeast heterologous expression with high metal concentrations (2.0mM Zn, 1.6mM Co and 5.0mM Mn). Zn and Co sensitive yeast strain \( zrc1cot1 \) and Mn sensitive yeast strain \( pmr1 \) were transformed with MTP1-4 and the empty vector \( piUGpd \). The strains were all grown for 3 days and equalize at \( OD \ 600nm \) to 1.0. A four-fold dilution was done and the yeast spotted on SD-Uracil media.
Figure 3.8 Expression of MTP1-4 in yeast *zrc1cot1*, *Pmr1* strains and wild type background. A- MTP1 in *zrc1cot1*, *pmr1* mutant strains and WT strains; B- MTP2, MTP3, MTP4 in *zrc1cot1* mutant strain; C- MTP2, MTP3, MTP4 in *pmr1* mutant strain; D- MTP2, MTP3, MTP4 in WT strain. Short primers of sizes between 100 and 200 bp was used for the amplification (MTP1- 120bp, MTP2- 180bp, MTP3- 160bp, MTP4- 120bp). All the strains both mutants (*zrc1cot1* and *pmr1*) and the wild type transformed with MTP1-4 showed expression determined by RT-PCR.
3.2.3 Evaluation of metal tolerance of MTP proteins by expression in wild type yeast

The wild type yeast (BY4741) was transformed with MTP1-4; this was to evaluate the respective metal tolerance of these transformed yeast strains. Transformed strains were grown in metal rich SD-U media for 24 hours to assess their metal tolerance. A control experiment was carried out without metal and showed no difference between the expression strains and the positive control as determined by optical density measurements (Fig. 3.9). At 20mM and at 40mM Zn, MTP1 showed a slight increase in cell density, indicating increased Zn tolerance compared to other strains with significant difference (P<0.05) compared to MTP2-4 (Fig. 3.10). The Mn transport genes MTP2-4 all showed an increase in Mn tolerance at 20mM and 40mM Mn with significant difference (P<0.0001) compared to MTP1 (Fig. 3.11), whereas MTP1 and MTP3 did not show significant increase in Co tolerance (Fig. 3.12). The measurement of biomass dry weight of the strains also indicated corresponding metal tolerance by the respective MTP proteins (Fig. 3.13-3.16). At 20mM Zn, MTP1 showed significant increase in Zn tolerance (P<0.05) compared to MTP2-4 (Fig 3.14). MTP2-4 were observed to show significant increase (P<0.01) in Mn tolerance at 20mM of Mn compared to MTP1 (Fig 3.15). Co tolerance was observed by MTP1 as it showed significant increase (P<0.0001) compared to MTP2-4 (Fig 3.16). Increase in specific metal tolerance for the different MTPs further confirms the heterologous characterization of the MTPs in yeast.
Figure 3.9 MTP yeast over-expression strains (wild type background) in medium treated with no metal (Control). SD-U yeast medium was used for the growth and growth period was 24 hours. PC- positive control. Data are means (±) SE of three replicates cultures per strain.
Figure 3.10 Metal tolerances of MTP yeast over-expression strains (wild type background) in medium treated with (A) 20mM Zn and (B) 40mM Zn. SD-U yeast medium was used for the growth and growth period was 24 hours. PC- positive control. Data are means (±) SE of three replicates cultures per strain. * (P<0.05) denotes significant difference between MTP1 and MTP2-4. ** (P<0.01) denotes significant difference between MTP1 and MTP2-4.
Figure 3.11 Metal tolerances of MTP yeast over-expression strains (wild type background) in medium treated with (A) 20mM Mn and (B) 40mM Mn. SD-U yeast medium was used for the growth and growth period was 24 hours. PC- positive control. Data are means (±) SE of three replicates cultures per strain. *** (P<0.0001) denotes significant difference between MTP2-4 and MTP1.
Figure 3.12 Metal tolerance of MTP yeast over-expression strains (wild type background) in medium treated with (A) 10mM Co and (B) 20mM Co. SD-U yeast medium was used for the growth and growth period was 24 hours. PC- positive control. Data are means (±) SE of three replicates cultures per strain.
Figure 3.13 Biomass dry weight of MTP yeast over-expression strains (wild type background) in medium treated with no metal (Control). SD-U yeast medium was used for the growth and growth period was 3 days. PC- positive control. Data are means (±) SE of three replicates cultures per strain.
Figure 3.14 Biomass dry weights of MTP yeast over-expression strains (wild type background) in medium treated with (A) 5mM Zn and (B) 20mM Zn. SD-U yeast medium was used for the growth and growth period was 3 days. PC- positive control. Data are means (±) SE of three replicates cultures per strain. * (P<0.05) denotes significant difference between MTP1 and MTP2-4.
Figure 3.15 Biomass dry weights of MTP yeast over-expression strains (wild type background) in medium treated with (A) 10mM Mn and (B) 20mM Mn. SD-U yeast medium was used for the growth and growth period was 3 days. PC- positive control. Data are means (±) SE of three replicates cultures per strain. ** (P<0.01) denotes significant difference between MTP2-4 and MTP1.
Figure 3.16 Biomass dry weights of MTP yeast over-expression strains (wild type background) in medium treated with (A) 10mM Co and (B) 20mM Co. SD-U yeast medium was used for the growth and growth period was 3 days. PC- positive control. Data are means (±) SE of three replicates cultures per strain. *** (P<0.0001) denotes significant difference between MTP1 and MTP2-4.
3.2.4 Assessing the cellular metal uptake by MTP1-4 yeast over-expression strains

Microorganisms have several mechanisms of resisting metal toxicity and one of these mechanisms is that they efflux excess metal ions into the outer environment through the secretory pathway or efflux pumps (Costa and Franca, 2003; Worms et al., 2006; Levy et al., 2008). The analysis of cellular metal by these transformed yeasts was considered vital to assess their metal uptake ability. Transformed yeast strains were cultured in SD-U yeast growth medium treated with 10mM Mn and 5mM Zn and were allowed to grow for 3 days, after which they were harvested, and washed with 1mM EDTA to remove cell wall-bound metal, before analysis by ICP-AES. However, at 10mM Mn, MTP 2-4 yeast strains did not show significant difference in Mn content compared to MTP1 and empty plasmid control. Likewise, at Zn 5mM, there was no significant difference in Zn content between MTP1 and MTP2-4 and empty plasmid control (Fig. 3.17).
Figure 3.17 A-Cellular Mn content of yeast over-expression strains treated with 10mM Mn. B- Cellular Zn content of yeast over-expression strains treated with 5mM Zn. PC- positive control. Data are means (±) SE of three replicates cultures per strain.
3.3 Discussion

3.3.1 The role of metal tolerance proteins (MTPs) in metal transport

CDFs are predicted to transport several metal ions which includes Zn$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Cd$^{2+}$, Fe$^{2+}$ and Co$^{2+}$ (Anton et al., 1999; Montanini et al., 2007; Munkelt et al., 2004; Persans et al., 2001; Williams and Pittman, 2010). As earlier mentioned, these transporters play a significant role in the transport of specific metal ions from the cytoplasm to other intracellular organelles (Blaby-Haas and Merchant, 2012; Peiter et al., 2007) thus mediating ion homeostasis in the cells.

Zn-CDFs have been characterized in several plants. In *Arabidopsis thaliana* and *Arabidopsis halleri*, the metal tolerance protein MTP1 is known to play a role in sequestration of extreme concentration of Zn in the vacuole (Ricachenevsky et al., 2013; Drager et al., 2004; Talk et al., 2006; Gustin et al., 2011), while AtMTP3, like AtMTP1, has been proposed to play major role in Zn$^{2+}$ transport and sequestration (Arrivault et al., 2006; Desbrosses-Fonrouge et al., 2005; Kramer, 2005; Kobae et al., 2004). Unlike AtMTP1, AtMTP3 could also have high affinity for the transport of Zn$^{2+}$ and Co$^{2+}$ (Arrivault et al., 2006; Kobae et al., 2004), and the fact that AtMTP3 transports Co$^{2+}$ distinguishes the function of this protein.

This Zn transport and sequestration role by an MTP1 orthologue is also reported and confirmed in this Chapter. *C. reinhardtii* MTP1 and MTP3 functionally characterized by yeast heterologous expression using the Zn sensitive mutant yeast strain zrc1cot1 displayed Zn tolerance activity, suggestive of Zn$^{2+}$ transport, with CrMTP1 having a strong tolerance activity. The Zn tolerance activity was observed at fairly high Zn concentrations which were 1.5 mM and 2.0 mM (Fig.3.7b and c). This strongly supports the fact that MTP1 proteins throughout green plants and algae play a significant role in transport and sequestration of high concentrations of Zn, as earlier demonstrated for MTP1 proteins in
other plants. However, the Zn tolerance activity shown by CrMTP3 was unexpected based on the sequence and phylogenetic analysis, and identification as a Mn-CDF. It therefore shows that CrMTP3 could have a broad substrate range for metal transport. Furthermore, the characterisation of CrMTP1 in this project revealed Co activity as well, though somewhat weak (Fig. 3.7b and c). However, CrMTP1 did not show any activity for Mn, which is similar to OsMTP1 and in common to other Zn-CDFs as reported by Menguer et al. (2013). Characterization of MTP1 and MTP3 orthologues in other plants have also displayed similar transport activities for Zn$^{2+}$ and Co$^{2+}$; including *Nicotiana glauca* NglauMTP1 (Shingu et al., 2005), *Hordeum vulgare* HvMTP1 (Podar et al., 2012), *Arabidopsis thaliana* AtMTP3 (Arrivault et al., 2006). CrMTP1 and CrMTP3 are therefore predicted to have some role in Co$^{2+}$ transport within the cell. These metal transporter proteins could also mediate the sequestration of Co$^{2+}$ in the vacuole and could also play a ‘housekeeping’ role by helping distribute Co to the organelles when needed.

In addition, members of the CDF metal transport family are predicted to play a role in the transport and regulation of Mn, for example, AtMTP8-11 (Blaby-Haas and Merchant, 2012; Pittman, 2005; Williams and Pittman, 2010; Hanikenne et al., 2005). *C. reinhardtii* MTP2-4 have previously been proposed to play roles in Mn$^{2+}$ transport (Blaby-Haas and Merchant, 2012; Maser et al., 2001) based on the gene sequence and phylogenetic analysis of these proteins, which is similar with other plants Mn-CDF genes. The functional characterization of MTP2-4 by yeast heterologous expression using a Mn sensitive yeast strain *pmr1*, confirmed that MTP2-4 equally exhibited a strong tolerance activity for Mn at all concentrations of Mn tested. Many Mn-CDF genes have been discovered from phylogenetic analysis, and these genes are predicted to be Mn specific. The surprising thing was that one of these CDFs could transport Mn$^{2+}$ and Zn$^{2+}$ as discovered in this study. CrMTP3 was observed to show affinity for both Zn$^{2+}$ and Mn$^{2+}$; this has not been reported previously for a Mn-CDF, thus making it novel, though further biochemical studies are required to confirm this finding.
3.3.2 Metal tolerance and uptake by MTPs in wild type yeast

The study also examined whether the functions of these metal transport proteins could be observed by over-expression in the wild type yeast which already contains the endogenous Zn and Mn transport genes, or whether CrMTP action would be masked. The wild type yeast strains however, showed significant difference in their Zn tolerance; MTP1 strains showed significant increase in Zn tolerance compared to other yeast strains with 20 mM Zn. Strains treated with 5mM Zn did not show any significant difference in Zn tolerance, probably due to the concentration of Zn which might have been less than the concentration required to cause toxicity. Likewise, MTP2-4 yeast expression strains showed significant increase in Mn tolerance compared to other strains at 20mM and 40mM Mn. The MTP2-4 yeast strain remarkable tolerance to Mn further confirms the predicted Mn$^{2+}$ transport role of MTP2-4 (Hanikenne et al., 2005; Delhaize et al., 2007; Allen et al., 2007). Although uptake of Zn and Mn was tested, there was no significant increase in Zn or Mn uptake by MTP-expressing strains at 5 mM Zn or 10mM Mn, probably due to the low metal concentration which might have not been taken up at sufficiently high concentration; higher Zn or Mn concentration maybe would have enhanced higher Zn and Mn uptake by the cells.

3.3.3 Protein similarities/diversities and the metal transport role of MTPs

MTPs have been reported to display similarities in metal specificity (Maser et al., 2001) even among some putative protein in plants and animals. Sequence and phylogenetic analysis indicated that C. reinhardtii MTP1 is homologous to Arabidopsis thaliana AtMTP1- AtMTP4 as well as S. cerevisiae Zrc1 and Cot1. However, the similarity displayed by these metal transport proteins does not suggest that one could replace the other. In the case of AtMTP1 and AtMTP3, the deletion of AtMTP1 or AtMTP3 led to Zn sensitivity, demonstrating that these proteins are not redundant despite having similar structural and metal transport characteristics, their role may not be swapped (Kobae et al., 2004; Kawachi et al., 2009); this therefore shows the uniqueness of each protein.
regarding its metal transport characteristics. ShMTP1-5 and *C. reinhardtii* MTP2-4 are also predicted to be homologous to each as they display similar metal ion transport characteristics (Delhaize et al., 2003; Pittman 2005); their Mn transport characteristics could be linked to their protein or DNA sequence similarity.

In this chapter, *C. reinhardtii* MTP1-4 have been characterized and there is therefore a need to examine their structural similarities and probably link that to their metal transport roles. The transmembrane helices prediction shows diversity and similarities among the MTP group. MTP1 displayed a significant difference in the transmembrane helices position for the last two transmembrane domains compared to MTP2-5 with the histidine stretch observed in MTP1 and not in MTP2-4, the internal loops for MTP1 showed uniqueness as the first four loops were observed to be located close to each other and the last two located at the far end which is similar with AtMTP1 and OsMTP1 except for the fact that the His loop in CrMTP1 is much longer compared to AtMTP1 and OsMTP1 (Fig. 3.4). The His-rich loop extends between the TM4 and TM5, and is predicted to play a significant role in metal ion specificity of the protein and mediates metal ion transport as well (Shingu et al., 2005; Kawachi et al., 2008; Podar et al., 2012; Ricachenevsky et al., 2013). A similar position histidine-rich region position is predicted for the ZnT sub-family in mammals, where several several histidine domains are harbored in a long cytoplasmic loop extending between TM4 and TM5, and this region is also predicted to have a Zn-binding property (Palmiter et al., 1995). In the MTP structure, major sites such as conserved residue stretches and specific TMDs could play a significant role in substrate specificity (Kawachi et al., 2012). TMDs I, II, V and VI are predicted to contain major polar charged residues which play a significant role in metal transport in MTP1 (Gaither and Eide, 2001). The his–rich loop is predicted to be actively involved in the determining the specificity of metal transport by the protein; hence could act as a chaperone detecting the metal ion to be transported (Kawachi et al., 2008; Podar et al., 2012). The role of the His loop has further been confirmed as the removal of the loop resulted in a non-functioning *Thlaspi caerulescens* MTP protein (Kim et al., 2004). AtMTP1 protein also has six
cysteine (Cys) residues and some of the residues play a vital role in substrate transport. The major residues for Zn–binding are conserved in most CDF family protein (Kawachi et al., 2012).

The Mn-CDF proteins also showed similarities in protein structure; CrMTP2-4 displayed similarities in TMD positions compared with other plant Mn-CDFs protein like AtMTP8 and 9 (Fig. 3.5). The protein structure similarities observed seems to be common among plant and algae Mn-CDFs. The diversities and similarities between the MTP sequences are obviously responsible for the differences and similarities showed by these metal transport protein in their metal specificity. This has improved our understanding of the principle behind the metal specificity displayed by these metal transporters and also promotes the uniqueness of these proteins in their respective functions.
4.0 Screening of CrMTP4 over-expression strains for Mn and Cd tolerance and uptake

4.1 Introduction

The potential of using plants and algae for metal bioremediation has triggered the transformation of plants and microalgae with metal homeostasis genes in tolerance, uptake, sequestration, transport and chelation of metals (Cia et al., 1999; Antosiewicz et al., 2014). Several metal transport genes have been over-expressed in plants including CDF metal transporters. Studies have shown that the transformation of plants and algae with metal transporters genes sometimes gives the expected result and sometimes produces results contrary to what was expected (Antosiewicz et al., 2014; Cia et al., 1999). For instance, over-expression of genes may trigger a metal uptake mechanism as expected (Thomas et al., 2003; Martinez et al., 2006; Korenkov et al., 2007; Gorinova et al., 2007); and could also cause a decrease in metal uptake and accumulation which is unexpected (Li et al., 2004; Wojas et al., 2008; Grispen et al., 2011; Barabasz et al., 2012). Though the reasons for the unexpected results might not be fully understood, but partly might be as a result of the fact that the foreign gene inserted into the genome could alter the physiological and biochemical pathways of the organism or might be localised or expressed differently in the foreign organism (Antosiewicz et al., 2014). This however, has been one of the challenges of over-expressing metal transport genes in plants or algae for enhanced metal tolerance and uptake. Notwithstanding, several studies have been done on the over-expression of MTPs in plants for enhanced metal transport (Ricachenevsky et al., 2013) and the expected result of enhanced metal tolerance, transport and accumulation was achieved. For example, PtdMTP1 over-expressed in Arabidopsis showed enhanced Zn tolerance (Blaudez et al., 2003). In another study, *N. goesingense* MTP1 protein over-expressed in Arabidopsis showed enhanced Zn accumulation in roots, though less accumulation in the shoot (Gustin et al., 2009).
As discussed in Chapter 3, *C. reinhardtii* MTP genes have not been previously characterized. By using yeast expression, it was shown in Chapter 3 that MTP1 was a Zn-CDF and MTP2-4 were Mn-CDFs. Over-expressing these proteins in *C. reinhardtii* for enhanced metal tolerance was therefore considered as the next step to evaluate the potential of algal genetic engineering for the bioremediation and treatment of metal-rich wastewater. Attempts to over-express MTP1 were unfortunately unsuccessful and will not be discussed here. In this Chapter, MTP4 was over-expressed in *C. reinhardtii* to attempt to enhance metal tolerance and uptake. This study was done to examine if over-expressing this Mn tolerance protein of the CDF metal transport family in *C. reinhardtii* could enhance metal tolerance and uptake.
4.2 Results

4.2.1 Over-expression of MTP4 in C. reinhardtii

Full length CrMTP4 genomic DNA encoding the CrMTP4 gene (3,067 bp) including exons and intron, and some UTR sequence was amplified by PCR then was ligated into the cloning vector pGEM-T Easy, then it was digested, purified and ligated into the Gateway entry vector pENTR1A and then into Gateway destination vector pH2GW7 by recombination using clonase, as shown in Fig. 4.1. CrMTP4 in pH2GW7 was under the control of the 35S promoter for over-expression into C. reinhardtii cells (11/32C) and selection on hygromycin. After the plasmid was transformed into 11/32C strain by biolistic bombardment and selected on hygromycin plates, 3 independent colonies (lines) were isolated and characterised further. Semi-quantitative RT-PCR analysis of these lines to check the expression of CrMTP4 was performed compared to wild type. The three lines showed higher level of MTP4 expression compared to the wild type, relative to the constitutive control gene RACK1, with the highest expression in line C (Fig 4.2).
Figure 4.1 Plasmid generated for *C. reinhardtii* MTP4 transformation. CrMTP4 was first cloned into pGEMT-Easy (3015bp), then cut at the EcoR1 sites, purified and cloned into pENTR1A (2717bp) and finally cloned into pH2GW7 (11522bp) using the Gateway recombination cloning technique for transformation into *C. reinhardtii*. 
Figure 4.2 RT-PCR analysis of CrMTP4 expression in the wild type and three MTP4 over-expression strains (named A, B and C). Expression is compared with RACK1, a constitutive control gene. Short primer of MTP4 (120bp) was used for the screening. RT-PCR was normalised with equal RNA addition. The PCR amplification cycle number used was 30.
4.2.2 Characterization of Mn and Cd tolerance in MTP4 over-expression strains.

The CrMTP4 over-expression strains showed normal growth compared wild type under non-stressed conditions, on the basis of chlorophyll content, cell count and cell density as determined by optical density (Fig. 4.5 and 4.6). CrMTP4 over-expression strains were screened for enhanced Mn tolerance. The strains were first treated with increasing concentrations of Mn as this was shown to be the specific substrate for MTP4 by yeast heterologous expression (Chapter 3). However, none of the CrMTP4 over-expression strain lines showed any difference in Mn tolerance compared to the wild type even at high concentrations (2mM) of Mn (Fig. 4.3, 4.5, 4.6). Chlorophyll content analysis and cell count to determine the cell density for Mn treated strains did not show any significant difference in tolerance between the strains and the wild type even at very high concentrations (1mM and 2mM) of Mn (Fig. 4.5, 4.6). However, screening with Cd did find that the MTP4 over-expression strains displayed significant tolerance for Cd at 300µM, 400µM and 500µM compared to wild type, which could not even grow in 500µM Cd (Fig. 4.11). Chlorophyll content analysis and optical density measurement to determine the cell density for Cd treated strains showed significant difference in tolerance between the strains and the wild type even at 300µM, 400µM and 500µM Cd concentrations (Fig. 4.9-4.11). Statistical analysis of the chlorophyll content and optical density of the strains was performed using a one way ANOVA and this showed significant increase in Cd tolerance at days 6 and 8 of growth (P<0.0001). The growth rate of the strains at 300µM and 400µM of Cd also showed a significant difference (P<0.05) between the strains and the wild type (Fig 4.12).
Figure 4.3 Growth and Mn tolerance of seven day old CrMTP4 over-expression strains at different concentrations of Mn. 1- strains grown in metal free medium, 2- strains treated with 1mM Mn, 3 strains treated with 2mM Mn. Representative image is shown.
Figure 4.4 Total chlorophyll (Chl a + b) content and cell count of wild type and CrMTP4 over-expression strains treated with no metal. A- Chlorophyll content of strains, B-cell count of strains. Data are means (±) SE of three replicate cultures per strain.
Figure 4.5 Total chlorophyll (Chl a + b) content and cell count of wild type and CrMTP4 over-expression strains treated with 1mM Mn. A- Chlorophyll content of strains, B-cell count of strains. Data are means (±) SE of three replicate cultures per strain.
Figure 4.6 Total chlorophyll (Chl a + b) content and cell count of wild type and CrMTP4 over-expression strains treated with 2mM Mn. A- Chlorophyll content of strains, B-cell count of strains. Data are means (±) SE of three replicate cultures per strain.
Figure 4.7 Growth and Cd tolerance of seven day old CrMTP4 over-expression strains at different concentrations of Cd. 1- strains grown in metal free medium, 2- strains treated with 400µM Cd, 3 strains treated with 500µM Cd. Representative image is shown.
Figure 4.8 Total chlorophyll (Chl a + b) content and optical density of wild type and CrMTP4 over-expression strains treated with no metal. A: Chlorophyll content of strains, B: optical density of strains. Data are means (±) SE of three replicate cultures per strain.
Figure 4.9 Total chlorophyll (Chl a + b) content and optical density of wild type and CrMTP4 over-expression strains treated with 300µM Cd. A- Chlorophyll content of strains. B-optical density of strains, Data is means (±) SE of three replicates cultures per strain. *** (P<0.0001) denotes significant difference between CrMTP4A-C and WT.
Figure 4.10 Total chlorophyll (Chl a + b) content and optical density of wild type and CrMTP4 over-expression strains treated with 400µM Cd. A- Chlorophyll content of strains. B-optical density of strains, Data is means (±) SE of three replicates cultures per strain. *** (P<0.0001) denotes significant difference between CrMTP4A-C and WT.
Figure 4.11 Total chlorophyll (Chl a + b) content and optical density of wild type and CrMTP4 over-expression strains treated with 500µM Cd. A- Chlorophyll content of strains treated with 500µM of Cd. B-optical density of strains. Data are means (±) SE of three replicates cultures per strain. The data at all the days are significant.
Figure 4.12 Growth rates of wild type and MTP4 over-expression strains determined at day 2 and 8. A- Strains treated with 300µM Cd, B-stains treated with 400µM Cd. Data are means (±) SE of three replicates cultures per strain. * (P<0.05) denotes significant difference between CrMTP4A-C and WT.
4.2.3 Metal content of *C. reinhardtii* MTP4 over-expression strains

To examine if the Cd tolerance displayed by strains corresponds with altered Cd uptake, cellular Cd concentration was measured. Although strains did not show a phenotype for Mn, Mn concentration was also measured. Strains were grown in medium containing 150µM Cd or 3mM Mn then cells were harvested after 7 days growth, EDTA washed to remove externally bound metals and prepared for intracellular metal analysis by ICP-AES. At this chosen concentration of Cd, there was no difference in tolerance between the over-expression strains and the wild type and it was assumed that any Cd uptake would have been negatively affected by Cd toxicity effects. However, there was no significant difference in Cd and Mn uptake as determined by cellular metal content between the over-expression strains and the wild type (Fig 4.13 and 4.14).
Figure 4.13 Intracellular Cd uptake of wild type and CrMTP4 over-expression strains at 150μM Cd. A- Cd uptake in atom per cell, B- Cd uptake in microgram per millilitre. Data are means (±) SE of three replicates cultures per strain.
Figure 4.14 Intracellular Mn uptake of wild type and CrMTP4 over-expression strains at 3mM Mn. A- Mn uptake in atom per cell, B- Mn uptake in microgram per millilitre. Data are means (±) SE of three replicates cultures per strain.
4.3 Discussion

The over-expression of CDF genes for enhanced metal tolerance and uptake started with Arabidopsis and has extended to various plants species including tobacco, *Brassica juncea*, *T. goesingense*, and *Oryza sativa* (Yuan et al., 2012). However, the over-expression of MTP/CDF genes for increased metal tolerance and uptake in any algae including *C. reinhardtii* has not to our knowledge yet been done, and therefore this study is first to over-express an MTP gene in *C. reinhardtii*.

Various plant genes in the Mn-CDF group including AtMTP8, ShMTP8 (formally known as ShMTP1), AtMTP9, AtMTP10, AtMTP11 have been predicted either by metal tolerance and uptake experiments or by phylogenetic analysis to play a significant role in Mn tolerance and transport (Gustin et al., 2011; Delhaize et al., 2003; Peiter et al., 2007). As CrMTP4 is also a Mn-CDF, much could be learnt regarding the evolution of this gene sub-family within the green lineage and to see how conserved the function of Mn-CDFs are between algae and plants. ShMTP8 provides Mn tolerance by vacuolar sequestration (Delhaize et al., 2003). Likewise, AtMTP11 plays a major role in Mn tolerance as displayed by heterologous experiments in yeast and by Mn sensitivity of the *MTP11* knock out, however, in this case not through direct vacuolar sequestration (Delhaize et al., 2007). The yeast expression results in Chapter 3 demonstrate that CrMTP4 shows much similarity with Mn-CDF genes from plants; however, the key difference as determined from the *C. reinhardtii* expression shown in this Chapter is the apparent Cd tolerance and transport activity by CrMTP4.

*C. reinhardtii* MTP4 has previously been predicted to relate to the sub-group of putative Mn-transporting CDFs (Hanikenne et al., 2005) and might be involved in vacuolar Mn storage. Furthermore, in Chapter 3, CrMTP4, along with CrMTP2 and MTP3, was confirmed to provide tolerance to Mn when expressed in yeast, presumably by causing
Mn$^{2+}$ transport. In this Chapter, when MTP4 was over-expressed in C. reinhardtii under the control of the constitutive 35S promoter, Mn tolerance phenotypes were not seen hence the role of increased abundance of MTP4 in Mn tolerance in this study was not significant. This could possibly be linked to the fact that CrMTP4 though predicted to play a role in Mn transport may not play a significant role in Mn tolerance in vivo; it may be involved in Mn homeostasis by transporting Mn to a specific location where needed but when increased in abundance that internal compartment may not be a great enough pool to provide significant tolerance. Also CrMTP4 may not have high capacity for Mn$^{2+}$ transport in C. reinhardtii. Allen et al. (2007) demonstrates that under situations of low Mn availability, out of all five Chlamydomonas MTPs tested, MTP4 shows the highest induction in response to Mn deficiency, indirectly agreeing with this assumption that MTP4 is indeed likely to be a high affinity transporter and therefore poorly suited to conditions of high Mn stress, and therefore unlikely to have a major role in Mn excess tolerance. It could also be that regulatory activities of other proteins such as other Mn transport proteins may mask the contribution to Mn tolerance by the over-expressed CrMTP4 compared to the wild type.

Mn tolerance in plants is achieved mostly by sequestration of Mn into the vacuole (Gonzalez and Lynch 1999; Fernando et al., 2006). Though there was no significant increase in the Mn tolerance of the CrMTP4 strains compared to the wild type, Mn uptake by CrMTP4 strains showed about a two-fold increase in two of the transformed lines (CrMTP4 B and CrMTP4 C) compared to the wild type (Fig 4.14). However, there was no significant difference in the Mn uptake increase compared to the wild type. It is worth considering the fact that maybe the high concentration of Mn (3mM) used could have had a negative effect on the cells and hence affected the uptake. A microscopic view of the cells stressed in 3 mM Mn showed about 50% of ruptured cells (result not shown) therefore the level of toxicity may have affected the uptake analysis negatively. Since Mn is one of the elements needed by cells for basic cellular metabolic activities (Williams and
Pittman, 2010) it could be proposed that Mn transport is more effective in cells not
exposed to toxic concentrations, though this is subject to confirmation by further study.
The CrMTP4 over-expression strains were also screened for Cd tolerance, as Cd is a
significant toxic metal of interest in this PhD study. Interestingly, compared to the wild type
CrMTP4 over-expression strains showed a significant increase in Cd tolerance (Fig 4.9-
Fig 4.11) particularly at 500 µM Cd (Fig 4.11and Fig. 4.7). As a Mn-CDF, MTP4 was not
predicted to play a role in Cd transport or tolerance, thererefore it was surprising to see that
the CrMTP4 over-expression strains showed significant Cd tolerance. However, the CDF
proteins have been predicted to play a role in the transport of a range of transition metals
including Cd, Zn, Co or Mn (Gaither and Eide, 2001); though there is yet no tested
evidence to prove that plants CDF proteins can transport Cd. The reason behind the
significant increase in Cd tolerance by the CrMTP4 C. reinhardtii strains is unclear.
Phylogenetic analysis of CrMTP4 and yeast heterologous expression predicts and
confirms that it is a Mn transporter (Chapter 3) and expression of MTP4 in wild type yeast
did not show evidence of Cd tolerance (data not shown). The Cd tolerance role displayed
could possibly be indirect and not possibly due to MTP4; it could be that some protein
regulatory activities in C. reinhardtii might be responsible for the increase in Cd tolerance
of the strains compared to the wild type (Antosiewicz et al., 2014). It may also be that Cd
efflux activity has been triggered by MTP4 over-expression which causes the flux of Cd
out of the cytoplasm and therefore gives Cd tolerance in this over-expression strain.
However, the fact that MTP4 is involved in metal homoeostasis and transport is another
positive view that could point to the fact the MTP4 is involved in Cd transport in these
strains. The result of Cd uptake did not correspond with the tolerance, as there was no
significant difference between the strains and the wild type (Fig. 4.13). However, this
could possibly be as a result of the concentration of Cd used which was 150µM. Probably
if a wider range of Cd concentrations were tested, there might have been evidence of Cd
increase; further work will therefore have to be done in the future to confirm these results.
Although sub-cellular localization of CrMTP4 was not examined in this study, the increase in Mn and Cd uptake and increased metal tolerance between the CrMTP4 strains and the wild type could suggest that CrMTP4 may be localized at the vacuolar membrane, and thus mediate Mn and Cd sequestration rather than efflux. It might be worthy of note to mention that localization of Mn-CDFs varies depending on their function; for example, ShMTP8 is also vacuolar localised and provides Mn sequestration (Delhaize et al., 2003). In contrast, Arabidopsis MTP11 appears to provide Mn tolerance via localisation at a different sub-cellular compartment which is either part of the Golgi (Peiter et al., 2007) or the pre-vacuolar compartment (Delhaize et al., 2007). CrMTP4 expression has been reported to be increased during Mn deficiency, which therefore suggests that MTP4 might possibly have a high affinity for Mn transport (Allen et al., 2007) which further confirms the significant role of this gene in Mn transport.

In summary, this study provides evidence of MTP4 function through over-expression in *C. reinhardtii*. Interestingly, this study has shown that CrMTP4 could play a significant role in Cd tolerance, the first demonstration of Cd tolerance mediated by MTP over-expression in algae and in plants.
5.0 Screening of AtHMA4 *Chlamydomonas reinhardtii* expression strains for Zn and Cd tolerance and uptake.

5.1 Introduction

Although Zn is considered an essential element for plants and animals, in excess it can pose a toxicity threat to these organisms (Kamal et al., 2004). In contrast, Cd is considered a non essential element to organisms (Lane et al., 2005; Xu et al., 2008) and tends to exert high toxicity. In order to maintain an intracellular and extracellular balance for these metals, organisms therefore have evolved mechanisms to regulate the amount of these metals in the cell. The mechanisms involved in Zn and Cd homeostasis are a complex one and are not fully understood, particularly in algae. As discussed in Chapter 1, metal transport proteins have been implicated in the transport of these metals into cellular compartments for sequestration and detoxification, and efflux of metals from the cell across the plasma membrane. In particular, members of the HMA family, including AtHMA4 have been predicted to play a role in the transport of Zn\(^{2+}\) and Cd\(^{2+}\), (Hussain et al., 2004; Gustin et al., 2009; Wong and Cobbett, 2009). HMAs are present in prokaryotes and eukaryotes including higher plants and green algae; in *Arabidopsis thaliana*, these metal transport proteins are grouped based on their substrate specificity; AtHMA1-4 are the divalent metal transport proteins, including for Zn\(^{2+}\) and Cd\(^{2+}\), and AtHMA5-8 transport univalent metal cations, such as Cu\(^+\) (Williams and Mills, 2005).

The important role of *A. thaliana* HMA4 in the transportation of Zn and Cd was first reported using yeast heterologous expression (Mills et al., 2003). Since then several studies have confirmed the role of AtHMA4 in transportation of Zn and also Cd (Hussain et al., 2004; Verret et al., 2004; Wong and Cobbett, 2009; Baekgaard et al., 2010). HMA4 is predicted to localize in the plasma membrane in both *A. thaliana* and *Arabidopsis halleri* (Verret et al., 2004; Courbot et al., 2007). Some previous studies have also examined the
function of AtHMA4 by expression in other organisms such as tobacco (Siemianowski et al., 2011) as well as yeast, but none of the plant HMA genes have previously been expressed in algae. Although HMA genes have been identified in the genomes of green algae including *C. reinhardtii*, algae HMA isoforms are yet to be characterised. In this chapter, the Zn and Cd tolerance role of AtHMA4 expressed in *C. reinhardtii* was evaluated and the results will be discussed.
5.2 Results

5.2.1 Protein sequence analysis of HMA genes

Protein sequence comparisons of HMA genes allow the identification of related gene sub-families, by phylogenetic analysis, and prediction of their substrate specificity. Comparing *C. reinhardtii* HMA genes with those known HMA genes from plants and yeast will help determine the possible substrate specificity. This will therefore help in determining whether it is appropriate to over-express a *C. reinhardtii* HMA or introduce a foreign plant HMA into *C. reinhardtii* for increased Cd and Zn tolerance and accumulation. Four HMAs have been identified in the *C. reinhardtii* genome (Merchant et al., 2006; Hanikenne et al., 2005). *C. reinhardtii* HMA1 (CrHMA1) clustered with HMA1 genes from other plants, including AtHMA1, AhHMA1 and OsHMA1 (Fig. 5.1). AtHMA1 and CrHMA1 share a specific Ser/Pro/Cys motif in the sixth transmembrane domain in place of the usual Cys-Pro-Cys/His/Ser motif and this characteristic is also shared by CoaT, a Co\(^{2+}\) transporter of *Synechocystis* (cyanobacterium), therefore it has been predicted that AtHMA1 could play a role in Co\(^{2+}\) transport (Cobbet et al., 2003). Considering the close similarity of CrHMA1 to AtHMA1 in the phylogenetic analysis, it might also be possible that CrHMA1 could have a role in Co\(^{2+}\) transport. Three other *C. reinhardtii* HMA genes that have been named CrCTP1, 2 and 3, are clustered close to AtPAA1, AtHMA5, AtHMA8 and ScPAA1 (Fig. 5.1), so this clustering with known Cu\(^{+}\) transporters suggests that these CTP genes are likely to encode Cu\(^{+}\) transporters (Fig 5.1). The known Cd and Zn transporters AtHMA2, AtHMA3, AtHMA4, AhHMA4 and NcHMA4 group together, as demonstrated previously (Williams and Mills, 2005).

None of the *C. reinhardtii* HMA genes appear to be grouped in the Cd transporter clade, hence the subsequent experiments in the chapter focuses on algal expression of AtHMA4, which is a known Cd transporter, rather than manipulation of one of the endogenous HMA transporters. In addition to the full-length AtHMA4 protein, the C-terminal tail of AtHMA4 has been shown to provide metal tolerance when expressed alone. For example, expression of the AtHMA4 C-terminal tail in yeast conferred tolerance to Zn and Cd
because of its metal binding characteristics (Bernard et al., 2004; Courbot et al., 2007; Baekgaard et al., 2010).
Figure 5.1 Phylogenetic tree of selected plant and yeast HMA subfamily of P-type ATPases. The tree shows that most of the genes are in the Cu, Zn, Co clades but none of the Chlamydomonas genes are in the Cd transporter clade. The HMA protein sequences used in the analysis are from *Arabidopsis thaliana* (At), *Oryza sativa* (rice Os), *C. reinhardtii* (Cr), *Arabidopsis helleri* (Ah), *Noccaea caerulescens* (Nc), *Saccharomyces cerevisiae* (Sc). Sequences were aligned using ClustalW2 (available at [http://www.ebi.ac.uk/Tools/msa/clustalw2/](http://www.ebi.ac.uk/Tools/msa/clustalw2/)) and the tree was generated using RaxmlGui 1.1 and visualized using Fig tree. Scale bar= 2 amino acid substitutions per site. Bootstrap percentage values are indicated at the tree nodes branches and indicate confidence in tree node positions.
5.2.2 Screening of *Chlamydomonas reinhardtii* AtHMA4 over-expression strains for Zn and Cd tolerance

Past studies have shown the characterization of transgenic HMA4 in other organisms including plants (Papoyan and Kochian, 2004; Mills et al., 2010; Hussain et al., 2004; Siemianowski et al., 2011) and yeast (Mills et al., 2003; 2010) confirming its role in Zn and Cd transport. However, AtHMA4 has not been characterised in *C. reinhardtii*. In this study, AtHMA4 *C. reinhardtii* over-expression strains were generated by the expression of either the full length (FL) AtHMA4 cDNA or cDNA encoding the C-terminal (CT) 473 amino acids of the protein. AtHMA4 FL and AtHMA4 CT cDNA in pH2GW7 plasmid under the control of the 35S promoter was transformed into *C. reinhardtii* cells (11/32C) previously by Dr Rachel Webster, and transformants were selected using hygromycin tolerance. RT-PCR analysis of the over-expression strains to confirm the expression of AtHMA4 FL and CT was performed in comparison to wild type using primers spanning the C-terminal region of AtHMA4 and with *RACK1* as a constitutive control gene. All the lines showed the presence of *AtHMA4* expression and the wild type did not (Fig. 5.2B).

The two different sets of AtHMA4 strains were screened for their Zn and Cd tolerance and uptake. In the screening experiment, the different AtHMA4 strains were treated with increasing concentrations of Cd (125\(\mu\)M, 150\(\mu\)M and 175\(\mu\)M) and Zn (300\(\mu\)M, 325\(\mu\)M, 350\(\mu\)M), and all strains showed metal tolerance phenotypes with all metal concentrations (Fig 5.2A, 5.4-5.9). AtHMA4 over-expression strains (all FL and CT lines) showed significant (P<0.0001) Zn tolerance at 300\(\mu\)M and 325\(\mu\)M compared to the wild type on the basis of optical density and chlorophyll content (Fig. 5.4 and 5.5). At 350 \(\mu\)M of Zn the strains showed significant tolerance while the wild type could not grow at this concentration (Fig. 5.6). A similar trend was observed for Cd tolerance; the AtHMA4 over-expression strains (all FL and CT lines) showed significant tolerance (P<0.0001) to Cd at 125\(\mu\)M and 150\(\mu\)M concentrations of Cd on the basis of optical density and chlorophyll content (Fig. 5.7 and 5.8). As with the highest concentration of Zn, at 175\(\mu\)M Cd, the wild
type could not grow whereas all the AtHMA4 strains still grew (Fig. 5.9). Growth rate measurements showed that all AtHMA4 over-expression strains had a significant increase in growth rate compared to the wild type in response to Zn toxicity, while the Cd toxicity showed no significant difference (Fig. 5.10). Cd concentrations below 125µM and Zn concentrations below 300µM could not display phenotypes (results not shown). Growth analysis was done at 2 day intervals from day 2 until day 8, although chlorophyll was analysed from day 4. Optical density values of the strains were measured to quantify cell density and cell counts were performed to quantify the growth rate. In the results, there was no difference with the strains in the absence of excess metals (Fig.5.11).
Figure 5.2  A-Phenotype of seven days old *C. reinhardtii* AtHMA4 over-expression strains [1-4 Full length (FL) and 5-8 C-terminus (CT)] at different concentrations of Zn and Cd. [1- wild type, CT1, CT2, CT3, CT4 treated with zinc 300µM; 2- wild type, CT1, CT2, CT3, CT4 treated with zinc 325µM; 3- wild type, CT1, CT2, CT3, CT4 treated with cadmium 125µM; 4- wild type, CT1, CT2, CT3, CT4 treated with cadmium 150µM; 5- wild type, FL1, FL2 treated with zinc 300µM; 6- wild type, FL1, FL2 treated with zinc 325µM; 7- wild type, FL1, FL2 treated with cadmium 125µM; 8- wild type, FL1, FL2 treated with cadmium 150µM]

B- Gel picture showing genotype of AtHMA4 over-expression strains. Expression is compared with *RACK1* a constitutive control gene. AtHMA4 primers with C-term overlapping region were used for the screening (45bp). RT-PCR was normalised with equal RNA addition. The PCR amplification cycle number used was 30.
Figure 5.3 Total chlorophyll (Chl a + b) content and optical density of wild type and *C. reinhardtii* HMA4 full length and C-terminus (FL and CT) over-expression strains. A- chlorophyll content of strains without metal treatment, B-optical density of strains without metal treatment. Data are means (±) SE of three replicates cultures per strain.
Figure 5.4 Total chlorophyll (Chl a + b) content and optical density of wild type and *C. reinhardtii* HMA4 full length and C-terminus (FL and CT) over-expression strains. A- chlorophyll content of strains treated with 300µM Zn, B-optical density of strains treated with 300µM Zn. Data are means (±) SE of three replicates cultures per strain. *** (P<0.0001) denotes significant difference between CT1-4, FL1-2 and WT.
Figure 5.5 Total chlorophyll (Chl a + b) content and optical density of wild type and C.reinhardtii HMA4 full length and C-terminus (FL and CT) over-expression strains. A- chlorophyll content of strains treated with 325µM Zn, B-optical density of strains treated with 325µM Zn. Data are means (±) SE of three replicates cultures per strain. *** (P<0.0001) denotes significant difference between CT1-4, FL1-2 and WT.
Figure 5.6 Total chlorophyll (Chl a + b) content and optical density of wild type and *C. reinhardtii* HMA4 full length and C-terminus (FL and CT) over-expression strains. A- chlorophyll content of strains treated with 350µM Zn, B-optical density of strains treated with 350µM Zn (WT did not grow at this concentration). Data are means (±) SE of three replicates cultures per strain. *** (P<0.0001) denotes significant difference between CT1-4, FL1-2 and WT.
Figure 5.7 Total chlorophyll (Chl a + b) content and optical density of wild type and *C. reinhardtii* HMA4 full length and C-terminus (FL and CT) over-expression strains. A- chlorophyll content of strains treated with 125μM Cd, B-optical density of strains treated with 125μM Cd. Data are means (±) SE of three replicates cultures per strain. *** (P<0.0001) denotes significant difference between CT1-4, FL1-2 and WT.
Figure 5.8 Total chlorophyll (Chl a + b) content and optical density of wild type and *C. reinhardtii* HMA4 full length and C-terminus (FL and CT) over-expression strains. A- chlorophyll content of strains treated with 150µM Cd, B-optical density of strains treated with 150µM Cd. Data are means (±) SE of three replicates cultures per strain. *** (P<0.0001) denotes significant difference between CT1-4, FL1-2 and WT.
Figure 5.9 Total chlorophyll (Chl a + b) content and optical density of wild type and *C. reinhardtii* HMA4 full length and C-terminus (FL and CT) over-expression strains. A- Chlorophyll content of strains treated with 175µM Cd, B-optical density of strains treated with 175µM Cd. (WT did not grow at this concentration). Data are means (±) SE of three replicates cultures per strain. *** (P<0.0001) denotes significant difference between CT1-4, FL1-2 and WT.
Figure 5.10 Growth rates of wild type and *C. reinhardtii* HMA4 full length (FL) and C-terminus (CT) over-expression strains determined at day 2 and 8. A- Strains treated with 300µM Zn, B-strains treated with 325µM Zn. ** (P<0.001) and *** (P<0.0001) denotes significant difference between CT1-4, FL1-2 and WT.
Figure 5.11 Growth rate of wild type and *C. reinhardtii* HMA4 full length (FL) and C-terminus (CT) over-expression strains determined at day 2 and 8. A- Strains treated with 125µM Cd, B-strains treated with 150µM Cd. Data are means (±) SE of three replicates cultures per strain.
5.2.3 Metal content of *Chlamydomonas reinhardtii* AtHMA4 over-expression strains

The metal toxicity resistance observed in these strains led to the question as to whether the significant level of Zn and Cd tolerance displayed by the strains could be linked to the ability to efflux metal ions out of the cell or internally sequester the metals. Intracellular metal quantification by ICP-AES was carried out to determine the metal content in the cells. Strains were treated with 300µM Zn and 150µM Cd, after 7 days growth, cells were harvested, EDTA-washed and prepared for intracellular metal analysis. AtHMA4 FL1 and CT3 lines showed significant increase (P<0.05) in Cd content compared to the wild type (Fig. 5.12), while FL2, CT1-3 lines showed an increase in Zn content compared to the wild type (Fig. 5.13). In addition to Cd and Zn, the concentrations of other trace metals were quantified. Mn content was observed to increase (P<0.001) significantly in FL1 and CT4 lines compared to the wild type (Fig. 5.14). FL1, CT1 and CT5 lines showed significant (P<0.001) increase in Cu content compared to wild type, as did the CT3 line (P<0.05) (Fig. 5.17). In contrast, there was no significant difference in Fe uptake between any of the the AtHMA4 strains and the wild type (Fig. 5.16).
Figure 5.12 Intracellular Cd uptake of wild type and *C. reinhardtii* HMA4 full length (FL) and C-terminus (CT) over-expression strains at 150µM Cd. A- Cd uptake in atom per cell, B- Cd uptake per optical density. * (P<0.05) denotes significant difference between engineered strains (CT1-4, FL1-2) and WT.
Figure 5.13 Intracellular Zn uptake of wild type and *C.reinhardtii* HMA4 full length (FL) and C-terminus (CT) over-expression strains at 150µM Zn. A- Zn uptake in atom per cell, B- Zn uptake per optical density. ** (P<0.001) and * (P<0.05) denotes significant difference between engineered strains (CT1-4, FL1-2) and WT.
Figure 5.14 Intracellular Mn uptake of wild type and *C. reinhardtii* HMA4 full length (FL) and C-terminus (CT) over-expression strains at 150µM Mn. A- Cd uptake in atom per cell, B- Mn uptake per optical density. ** (P<0.001) and * (P<0.05) denotes significant difference between engineered strains (CT1-4, FL1-2) and WT.
Figure 5.15 Intracellular Cu uptake of wild type and *C. reinhardtii* HMA4 full length (FL) and C-terminus (CT) over-expression strains at 150µM Cu. A- Cd uptake in atom per cell, B- Cu uptake in microgram per million cells, C-Cu uptake per optical density. ** (P<0.001) and * (P<0.05) denotes significant difference between engineered strains (CT1-4, FL1-2) and WT.
Figure 5.16 Intracellular Fe uptake of wild type and *C. reinhardtii* HMA4 full length (FL) and C-terminus (CT) over-expression strains at 150µM Fe. A- Fe uptake in atom per cell, B- Fe uptake in microgram per million cells. C-Fe uptake per optical density.
5.3 Discussion

5.3.1 The role of HMA4 in Zn and Cd translocation

As earlier discussed, HMAs are transmembrane proteins that are known to transport transition metals. The main function of HMA4 in *Arabidopsis thaliana* is the transport of $\text{Zn}^{2+}$ and $\text{Cd}^{2+}$ from the cell (Hussain et al., 2004; Verret et al., 2004; Mills et al., 2005; Wong and Cobbett, 2009). The screening of *C. reinhardtii* AtHMA4 full length over-expression strains showed significant increase in Zn tolerance compared to the wild type. At 300$\mu$M and 325$\mu$M Zn the strains showed a significant ($p<0.0001$) increase in growth and chlorophyll content (Fig 5.4 and Fig 5.5). Interestingly, at 350$\mu$M Zn the wild type could not withstand the toxicity of the metal and hence could not grow at all, while the transgenic strains grew, although they were stressed (Fig 5.6). High tolerance of the AtHMA4 strains to Cd was also observed at 125$\mu$M and 150$\mu$M Cd compared to the wild type, while the wild type never showed any growth at 175$\mu$M Cd yet the AtHMA4 strains showed substantial growth. It is very clear that the tolerance of Zn and Cd in the *C. reinhardtii* AtHMA4 over-expression strains is a function of AtHMA4. Several other studies have shown the role of AtHMA4 in Zn tolerance and transport (Hanikenne et al., 2008; Bernard et al., 2004; Papoyan and Kochian, 2004) while the role of AtHMA4 in Cd transport has been previously reported (Craciun et al., 2012). Furthermore, a similar role of AtHMA4 has been replicated in *A. halleri* (Hanikenne et al., 2008; Courbot et al, 2007).

In another study, ectopic over-expression of AtHMA4 was shown to improve root growth in high concentrations of Zn, Cd and Co (Verret et al., 2004). Likewise, over-expression of AhHMA4 in yeast cells induced Zn and Cd tolerance by reducing the Cd and Zn content of in the cells concluding that AhHMA4 could be playing the role of effluxing Zn and Cd out of the cell (Courbet et al., 2007).

The cellular Cd and Zn content of most of the AtHMA4 *C.reinhardtii* strains was significantly increased ($p<0.05$) compared to the wild type (Fig 5.12 and Fig 5.13). Interestingly, Mn and Cu content also showed significant increase ($p<0.05$) compared to
the wild type (Fig 5.14, Fig 5.15). Although HMAs have been predicted to transport Cu, there is yet no report specifically attributing a Cu transport role to HMA4. A Mn transport characteristic of HMA4 has not been reported at all. However, it is difficult to conclude that increased Mn content in these strains was directly due to AtHMA4 expression or an indirect consequence of altered Zn homeostasis; further studies need to be done to confirm this. Fe content by the strains showed no difference with the wild type, which confirms that AtHMA4 does not play any role in Fe transport (Fig 5.16).

Interestingly, *C. reinhardtii* AtHMA4 C-terminal over-expression strains showed significant (p<0.0001) increase in Zn and Cd tolerance compared to the wild type just like the full length over-expression strain. This was not surprising, but the surprising aspect was that AtHMA4 CT strains tolerated Zn and Cd equally like the full length over-expression strain compared to the wild type. Metal content results also followed the trend with significant (p<0.05) increase in Cd, Zn, Mn and Cu content compared to the wild type. Although Cu content showed significant (p<0.05) increase compared to wild type (Fig 5.15), increased Cu uptake was observed in the full length strains compared to the C-terminus strains. This could be because AtHMA4 C-terminus does not have strong Cu affinity like it does with Zn and Cd. Expression of the AtHMA4 C-terminus in yeast previously showed tolerance to Cd and Zn (Bernard et al., 2004; Courbot et al., 2007; Baekgaard et al., 2010). The C-terminus of AtHMA4 is thought to act as a metal-binding peptide, which could likely affect the level of metal uptake when expressed in plants (Craciun et al., 2012). Interestingly this study has shown the Zn, Cd, Cu and Mn tolerance and transport characteristic of AtHMA4 C-terminus in *C. reinhardtii*. However, further research is needed to understand fully the role played by this region of the transport protein in metal tolerance. In a similar study, the expression of mothbean pyrroline-5-carboxylate synthetase (P5CS) gene in *C. reinhardtii* showed enhanced tolerance and binding of Cd to GSH predicted to be triggered by increased proline-dependent cytosolic GSH induction compared to the wild type (Siripornadulsil et al., 2002). P5CS plays a key role in the synthesis of proline and proline is also predicted to play a role in osmotic stress tolerance in plants, this could probably
play a role in Cd tolerance as well as other toxic metals (Kamoun et al., 1998). Rubinelli et al. (2002) showed that heterologous expression of a *C. reinhardtii* Cd- and Fe-induced gene H43 in the *S. cerevisiae* Fe uptake mutant *fet3fet4* resulted in a 2-fold increase in Fe accumulation per cell; H43 is a periplasmic protein synthesized in *C. reinhardtii* with metal binding characteristics. These studies clearly shows that proteins with metal binding characteristics could also play a role in metal tolerance.

The localization of HMA4 at the subcellular level in *A. thaliana* and *A. halleri* has been confirmed to be in the plasma membrane (Verret et al., 2004; Courbet et al., 2008) and in the root vascular pericycle layer at the tissue level (Verret et al., 2004; Hanikenne et al., 2008). Metals are moved out of the xylem parenchyma cells across the plasma membrane into the shoot tissues. Although the localisation of the AtHMA4 in *C. reinhardtii* was not shown in this study, increased Zn and Cd content suggests that it is not localised in the plasma membrane. AtHMA4 has the ability to play the role of an efflux pump, therefore helping to get rid of the excess Zn and Cd out of the cell, which is one of the mechanisms proposed for plants and yeast tolerance to high metal concentrations (Mills et al., 2005) but in this study AtHMA4 is presumed to play the role of sequestering the metal ions into the vacuole or another internal compartment for detoxification. This is predicted by the metal content measurement which indicated a high level of Zn and Cd uptake in the *C. reinhardtii* full length AtHMA4 expression lines compared to the wild type (Fig. 5.13 and 5.12). If AtHMA4 in *C. reinhardtii* was playing the role of effluxing metal ions out of the cell, the cellular Zn and Cd uptake level would probably be lower in the AtHMA4 expression strains compared to the wild type or would show no obvious difference. Hence it is predicted that the AtHMA4 is localised in the vacuole in *C. reinhardtii* since metal content in the over-expression strains are significantly higher than the wild type. This obviously differs from the phenotypes in plant and yeast over-expressinh AtHMA4 where metal concentrations were found to reduce compared to the controls indicating metal efflux (Mill et al., 2005; Siemianowski et al., 2014). Siemianowski et al. (2014) showed that AtHMA4 is localised at the plasma membrane of tobacco plants over-expressed with
AtHMA4 and Zn accumulation in the transformants was significantly lower than the wild type as a result of the efflux activity of the AtHMA4.

In this study, it was predicted that the full length AtHMA4 could have more metal tolerance and uptake role than the C-terminus considering the fact that the C-terminus is just a portion of the HMA4 transport protein. In contrast, it has been predicted that the C-terminus could play a role in regulating metal tolerance (Mills et al., 2010). Its role as a metal binding peptide could have effect on metal tolerance, hence playing an indirect role in metal tolerance. The heterologous expression of AtHMA4 full length and C-terminus separately in a yeast mutant sensitive to Zn and Cd displayed a significant increase in Zn and Cd tolerance (Mills et al., 2005). This role of AtHMA4 full length and the C-terminus in Zn and Cd tolerance is replicated in this study. In another study, the expression of the C-terminus of AtHMA4 in wild type tobacco treated with 0.5 or 5 µM Zn and 0.25µM Cd displayed Zn and Cd accumulation in roots and shoots (Siemianowski et al., 2011). This is consistent with the metal tolerance results shown in this study. It therefore confirms the significant role played by the full length and C-terminus of the AtHMA4 in Zn and Cd tolerance. However it has been demonstrated that the C-terminus may not play any significant role in Zn and Cd transport in plants considering the fact that it’s removal from the AtHMA4 full length did not affect the role of AtHMA4 in Zn and Cd transport (Mill et al., 2010). Although in another study, the removal of the C-terminus from AtHMA2 had a slight negative effect on the Zn transport (Wong et al., 2009). The reason for this may not be known but it has been suggested that this could be due to the difference in the structure of AtHMA2 and AtHMA4 C-terminus. AtHMA2 is observed to have a shorter C-terminal end (260 aa) whereas the size of the AtHMA4 C-terminus is predicted to be 470 aa (Mills et al., 2010). This could obviously contribute to the difference in the Zn and Cd tolerance role by these two metal transport proteins. Earlier studies suggest that though AtHMA4 and AtHMA2 have a significant role in the Zn nutrition in plants, it is also obvious that it might play a role in the transport of Zn into the xylem for onward transport to the shoot (Mills et al., 2005; Verret et al., 2004).
Screening of other HMAs for their metal tolerance and uptake role in *C. reinhardtii* is the future ambition for these studies. For example, this Chapter and past studies have predicted CrCTP1-3 to be Cu transporters from the monovalent metal transporter subfamily of HMAs (Hanikenne et al., 2005; Merchant et al., 2006), but none of these proteins have been functionally characterised. It is also fascinating to have AtHMA4 replicate the earlier predicted function in *C. reinhardtii*. The fact that metal tolerance by the *C. reinhardtii* AtHMA4 strains is not mediated by metal ion efflux out of the cell as in the case with plants and yeast but by proposed transport into the vacuole for sequestration and detoxification is interesting. This study is therefore novel considering the fact that this is the first study where AtHMA4 (full length and C-terminus) is screened in *C. reinhardtii* for Zn and Cd tolerance and uptake.
6.0 Comparing genetically engineered strains with natural strains for metal tolerance and uptake.

6.1 Introduction

Of all the thousands of algae species known so far, only a small number have been explored for their metal uptake and tolerance abilities (Mehta and Guar, 2005). Therefore taking maximum advantage of the vast diversity of algae strains for metal bioremediation studies could be a way forward to tackling the challenges posed by metal pollution. This is despite the fact that genetic manipulation of microalgae has been investigated for bioremediation of metals (Antosiewicz et al., 2014; Yuan et al., 2012; Siemianowski et al., 2011). Previous studies such as by Lessmann et al. (2000) and Doi et al. (2001) demonstrate that the use of natural strains from industrial wastewater, mine sites or naturally metal-rich sites could have great advantage for metal bioremediation and wastewater treatment. However, a side-by-side comparison of natural strains versus engineered strains with regard to metal tolerance and uptake has not been performed.

Metal bioremediation and sorption has been studied using algae from various natural habitats e.g. *Chlamydomonas acidophila*, *Chlorella* spp., *Scenedesmus* spp., *Sargassum natans*, *Fucus vesiculosus* (Mehta and Guar, 2005). Microalgae from waste sites are adapted over many generations to such pollutant-rich environments and therefore might be expected to perform better for metal bioremediation and wastewater treatment than non-adapted strains. However, there are environmental and physiological factors that can affect uptake and tolerance efficiencies therefore strains have to be evaluated under controlled conditions to determine the best strains (Chojnacka et al., 2005). Some of these organisms have been shown to tolerate extreme environmental conditions; for example *C. acidophila* is known to grow in and tolerate very metal-rich environments, and can accumulate metals from this environment (Lessmann et al., 2000; Doi et al., 2001). The mechanisms for metal tolerance are not always clear but may not always be due simply to
metal uptake and sequestration. For example *Parachlorella hussii* and *Parachlorella kessleri* have high tolerance to oxidative stress (Osundeko et al., 2013, 2014).

Exploring the adaptive mechanism of microalgae to extreme environmental conditions could be applied in the bioremediation of metals. *C. acidophila* is one example of a microalga that can thrive well in extreme environmental conditions. This organism tolerates highly acidic environments (Lessmann et al., 2000; Diol et al., 2001), which are often metal-rich (Nishikawa et al., 2003; Lessmann et al., 1999). Wastewater tolerant microalgae have also been discovered (Osundeko et al., 2013, McGinn et al., 2012). For example, Osundeko et al. (2013, 2014) identified two chlorophyte species *P. hussii*, and *Chlorella luteoviridis* that have high oxidative stress tolerance and therefore may also be more tolerant to metal stress, although this has not been tested previously. In this chapter some of these natural strains were screened for their metal tolerance and uptake ability and directly compared with the genetically engineered strains developed in this study to see which strains could perform the best in metal bioremediation activities.
6.2 Results

6.2.1 Screening of *C. reinhardtii*, *C. luteoviridis*, *P. kessleri* and *P. hussii* for metal tolerance

*C. luteoviridis*, *P. kessleri*, *P. hussii* and *C. reinhardtii* (used as a known non-metal tolerant control) were screened for their metal tolerance characteristics by testing Cd, Cu, Al and Zn. Chlorophyll content was used as a stress indicator as this was considered to be appropriate to screen for metal tolerance as most metals at high concentration cause reduction in chlorophyll biosynthesis and photosynthesis (Kobayashi et al. 2006; Vega et al. 2006). Chlorophyll content of these strains was determined every two days after treatment with four different concentrations of the four metals at increasing concentrations of 75µM, 100µM, 125µM and 150µM for Cd, Cu and Al, while 225µM, 250µM 270µM and 275µM were used for Zn. *P. hussii*, *P. kessleri* and *C. luteoviridis* showed significant increase (P<0.05) in Cd tolerance compared to *C. reinhardtii* at 100µM of Cd (Fig. 6.1). However, at a higher Cd concentration (150µM), *P. hussii* performed better compared to the other strains. All of the putative metal tolerant strains showed significant increase (P<0.05) in Cu tolerance at 75µM and 100µM Cu at day 8 compared to *C. reinhardtii*, but at 150 µM Cu *P. hussii* showed more tolerance (Fig. 6.2). All metal tolerant strains showed significant increase (P<0.05) for Al tolerance compared to *C. reinhardtii* (Fig. 6.3). *P. hussii* showed significant increase in Zn tolerance compared to *C. reinhardtii* and was observed to perform better than the other metal tolerant strains (Fig. 6.4). It should be noted that in the absence of excess metals, *P. hussii* had ≥ 35% increase in growth compared to *C. reinhardtii*, whereas, *P. kessleri* and *C. luteoviridis* showed ≥ 20% increase in growth compared to *C. reinhardtii* at day 8 (Fig. 6.1A).

Specific growth rate of strains treated with Cd showed significant increase (P<0.0001) in growth rate for *P. hussii*, *P. kessleri* and *C. luteoviridis* compared to *C. reinhardtii* (Fig. 6.5A). A similar trend was observed for the Cu and Al treated strains (Fig. 6.5B and Fig. 6.5C).
6.6A). The growth rate of strains in excess Zn also showed a significant increase (P<0.0001) for *P. hussii*, *P. kessleri* and *C. luteoviridis* compared to *C. reinhardtii* (Fig. 6.6B).
Figure 6.1 Chlorophyll content of natural strains treated with different concentrations of Cd. A-E Strains with no added metal (control) (A), with 75µM Cd (B), with 100µM Cd (C), with 125µM Cd (D), and with 150 µM Cd (E). C.r: *C. reinhardtii*, P.k: *Parachlorella kessleri*, P.h: *Parachlorella hussii*, C.l: *Chlorella luteoviridis*. Data are means (±) SE of three replicate cultures per strain. * (P<0.05) denotes significant difference between P.k, P.h, C.l and C.r (comparing P.k, P.h and C.l with C.r).
Figure 6.2 Chlorophyll content of natural strain treated with different concentrations of Cu.

A-D Strains treated with 75µM Cu (A), with 100µM Cu (B), with 125µM Cu (C), and with 150 µM Cu (D). C.r: C. reinhardtii, P.k: Parachlorella kessleri, P.h: Parachlorella hussii, C.l: Chlorella luteoviridis. Data are means (±) SE of three replicates cultures per strain. * (P<0.05) denotes significant difference between P.k, P.h, C.l and C.r. (comparing P.k, P.h and C.l with C.r).
Figure 6.3 Chlorophyll content of natural strain treated with different concentrations of Al.
A-D Strains treated with 75µM Al (A), with 100µM Al (B), with 125µM Al (C), and with 150 µM Al (D). C.r: *C. reinhardtii*, P.k: *Parachlorella kessleri*, P.h: *Parachlorella hussii*, C.l: *Chlorella luteoviridis*. Data are means (±) SE of three replicates cultures per strain. * (P<0.05) denotes significant difference between P.k, P.h, C.l and C.r. (comparing P.k, P.h and C.l with C.r).
Figure 6.4 Chlorophyll content of natural strain treated with different concentrations of Zn
A-D Strains treated with 225µM Zn (A), with 250µM Zn (B), with 275µM Zn (C), and with
300 µM Zn (D). C.r: *C. reinhardtii*, P.k: *Parachlorella kessleri*, P.h: *Parachlorella hussii*, C.l: *Chlorella luteoviridis*. Data are means (±) SE of three replicates cultures per strain. * (P<0.05) denotes significant difference between P.k, P.h, C.l and C.r (comparing P.k, P.h and C.l with C.r).
Figure 6.5 Growth rates of natural strains treated with different concentrations of metals.

A- Strains treated with 75µM - 150µM Cu. B- Strains treated with 75µM - 150µM Cd. C.r: C. reinhardtii, P.k: Parachlorella kessleri, P.h: Parachlorella hussii, C.l: Chlorella luteoviridis. Data are means (±) SE of three replicates cultures per strain. *** (P<0.0001) denotes significant difference between P.k, P.h, C.l and C.r (comparing P.k, P.h and C.l with C.r).
Figure 6.6 Growth rates of natural strains treated with different concentrations of metals

A- Strains treated with 75µM - 150µM Al. B- Strains treated with 225µM - 300µM Zn. C.r: *C. reinhardtii*, P.k: *Parachlorella kessleri*, P.h: *Parachlorella hussii*, C.l: *Chlorella luteoviridis*. Data are means (±) SE of three replicates cultures per strain. *** (P<0.0001) denotes significant difference between P.k, P.h, C.I and C.r (comparing P.k, P.h and C.I with C.r).
6.2.2 Cellular metal uptake of *C. reinhardtii, C. luteoviridis, P. kessleri* and *P. hussii*

Intracellular Cu and Al content of the natural strains was determined using ICP-AES. This was to ascertain if the high tolerance of these strains to the metals had any relationship with metal accumulation in the cell. The results show intracellular Cu and Al uptake in *C. reinhardtii, P. kessleri, P. hussii, and C. luteoviridis* (Fig. 6.7) at 50µM concentration of each metal. *P. hussi, P. kessleri* and *C. luteoviridis* showed significant (P<0.05) increase in Cu content compared to *C. reinhardtii*. However, *P. kessleri* showed significant (P<0.05) increase in Al content compared to *C. reinhardtii* and performed better than *P. hussi* and *C. luteoviridis*. Cd and Zn content of the natural strains were determined alongside with the genetically engineered strains for comparison and results are presented in the next segment of this chapter.
Figure 6.7 Cellular content of metals in the natural strains. A - Cellular content of Cu at 50µM, B - Cellular content of Al at 50µM. C.r: *C. reinhardtii*, P.k: *Parachlorella kessleri*, P.h: *Parachlorella hussii*, C.l: *Chlorella luteoviridis*. Data are means (±) SE of three replicates cultures per strain. In A - * (P<0.05) denotes significant difference between P.k, P.h, C.l and C.r. B - * (P<0.05) denotes significant difference between P.k and P.h, C.l, C.r.
6.2.3 Comparison of natural strains with genetically engineered strains for metal tolerance and uptake

The metal tolerance of *C. luteoviridis, P. kessleri* and *P. hussii* was compared with selected *C. reinhardtii* genetically modified (GM) strains over-expressing AtHMA4 (both full length, FL, and C-terminal tail, CT) and CrMTP4, along with the control (wild type) *C. reinhardtii*. The metals used for the comparison were Zn and Cd. The best-performing AtHMA4 FL and CT over-expression strains and the CrMTP4 over-expression strain showed high tolerance to Zn and/or Cd compared to the control *C. reinhardtii*, as also reported in Chapter 4 and 5. The results show Cd and Zn tolerance of both the natural and engineered strains at 150µM and 300µM concentrations of the metals (Fig 6.8). CrMTP4 strains were not included in the Zn tolerance analysis as they did not show tolerance to Zn when previously screened with the wild type (Chapter 4). *P. hussii, P. kessleri* and *C. luteoviridis* and all three GM strains (AtHMA4 FL1, CT1 and CrMTP4A) showed a significant increase (P<0.0001) in Cd tolerance compared to *C. reinhardtii* (Fig. 6.8B). In a comparison between the GM strains and natural strains, the natural strains showed increased (P<0.0001) Cd tolerance compared to the GM strains (Fig. 6.8B). A similar trend was observed for Zn tolerance as *P. hussii, P. kessleri* and *C. luteoviridis* all showed increased (P<0.0001) Zn tolerance compared to AtHMA4 FL1 and CT1, although these GM lines still showed increased Zn tolerance compared to *C. reinhardtii* (Fig. 6.8C).

Cd and Zn content was measured by ICP-AES in all strains. *P. hussii, P. kessleri* and *C. luteoviridis* showed increased Cd and Zn content compared to *C. reinhardtii* (Fig. 6.9). Likewise, AtHMA4 FL1 and CT1 GM strains showed significantly increased (P<0.001) in Cd and Zn content compared to wild type *C. reinhardtii*. In comparison between the natural strain and the GM strains, the natural strains showed significantly better performance (P<0.0001) with Cd uptake compared to the GM strains (Fig. 6.9A). However, in terms of Zn content, there was no significant difference between *P. hussii* and *C. luteoviridis* and the GM strains, but *P. kessleri* had increased Zn content compared to the GM strains (Fig. 6.9B).
Figure 6.8 Chlorophyll content of natural strains and genetically modified strains. A-C Chlorophyll content of strains with no added metal (A), with 150µM Cd (B) and with 150µM Zn (C). C.r: *C. reinhardtii*, P.k: *Parachlorella kessleri*, P.h: *Parachlorella hussii*, C.l: *Chlorella luteoviridis*, FL1: AtHMA4 full length over-expression line 1, CT1: AtHMA4 C-terminus over-expression line 1, CrMTP4 A: CrMTP4 over-expression line A. Data are means (±) SE of three replicates cultures per strain. *** (P<0.0001) and ** (P>0.01) denotes significant difference between natural strains (P.k, P.h, C.l) and GM strains.
Figure 6.9 Cellular metal uptake of natural strains and genetically modified strains. A- chlorophyll content of strains treated with 150µM Cd. C- chlorophyll content of strains treated 150µM Zn. C.r: C. reinhardtii, P.k: Parachlorella kessleri, P.h: Parachlorella hussii, C.l: Chlorella luteoviridis, FL1: AtHMA4 full length over-expression line 1, CT1: AtHMA4 C-terminus over-expression line 1. Data are means (±) SE of three replicates cultures per strain. A - ***(P<0.0001) denotes significant difference between natural strains (P.k, P.h, C.l) and GM strains. B - ** (P<0.01) denotes significant difference between P.k and GM strains (FL1, CT1). P.h and CT1.
6.2.4 Screening of *C. reinhardtii* cell walled and cell wall-less strains for metal tolerance

Cell walls of microalgae are able to bind metal ions and therefore the presence and characteristics of the cell wall could play a role in metal tolerance and uptake. In the light of this, *C. reinhardtii* cell walled and cell wall-less strains were screened for their tolerance to Cd, Cu, Al and Zn. Chlorophyll content of these strains treated with Cd, Cu, Al and Zn was determined at two day intervals. At 150µM of Cd, Cu and Al, and at 300µM Zn there was no significant difference between the walled and the walled-less strains (Fig. 6.10). Metal content measurement for these strains revealed no significant difference for 50µM concentration of Cd, and Al but for Cu and Zn uptake, the cell wall-less strain showed a significant increase in Zn content compared to the cell walled strain (Fig. 6.11).
Figure 6.10 Chlorophyll content of cell walled and cell wall-less *C. reinhardtii* treated with different metals. A-E Strains treated no metal (A), with 150µM Cd (B), with 150µM Cu (C), with 150µM Al (D), and with 300µM Zn (E). CW15: cell wall-less strain, 1132C: cell walled strain. Data are means (±) SE of three replicates cultures per strain.
Figure 6.11 Cellular metal content of *C. reinhardtii* cell walled and cell-less strains. A- Cellular Cu content of strains treated with 50 µM Cu. B- Cellular Al content of strains treated with 50 µM Al. C- Cellular Cd content of strains treated with 50 µM Cd. D- Cellular Zn content of strains treated with 100 µM Zn. CW15: cell wall-less strain, 1132C: cell wall strain. Data are means (±) SE of three replicates cultures per strain. * (P<0.05) denotes significant difference between wall-less and walled strain.
6.3 Discussion

6.3.1 Waste water adapted natural strains (*P. hussii*, *P. kessleri* and *C. luteoviridis*) show enhanced metal tolerance and uptake compared to AtHMA4 and CrMTP4 GM *C. reinhardtii* strains

Coping with the menace of metal pollution is very challenging; hence several studies using microalgae of both natural and genetically engineered strains have been geared towards remediating the environment from metal pollutants. Several studies have proven that algae and plants could be engineered to enhance metal bioremediation (Siripornadulsil et al., 2002; Cai et al., 1999; Mill et al., 2010; Verret et al., 2004; Courbet et al., 2008), but whether engineered strains could do better in metal tolerance and uptake than natural adapted strains was unknown. Several studies have demonstrated that algae could be genetically engineered and use potential tools for decontamination of the metal rich wastewater treatment (Cai et al., 1999; Siripornadulsil et al., 2002). It has been argued that GM microalgae would not be acceptable for use for commercial purposes as a result of regulatory restrictions and fear of environmental threats (Flynn et al., 2013). The safe handling of the GM strains to avoid environmental hazards could be another point of great concern. It is very clear that though the GM strains could be beneficial in metal bioremediation, their exposure to the environment could lead to risks of gene flow and competition against non-GM strains which could lead the extinction of natural strains of a particular organism in the affected habitat. Therefore physical and biological mechanisms must be put in place to prevent uncontrollable release of GM algae into the environment as this may pose a lot of problems (Rajamani et al., 2007).

Wastewater is a habitat that can pose a high level of toxicity to microalgae (Osundeko et al., 2014) as a possible result of several factors such as the source and wastewater type (Pittman et al., 2011). Possible factors could range from high heavy metal concentration, high level of ammonium concentration to high oxygen concentration (Wrigley and Toerien, 1990). However, microalgae with high tolerance and growth in wastewater have been
discovered (McGinn et al., 2012; Wu et al., 2012; Osundeko et al., 2013; 2014). These organisms are characterized by high biomass yield when grown in wastewater. *P. hussii* and *C. luteoviridis* are among some of the microalgae identified with the characteristic of tolerating and growing well in wastewater (Osundeko et al., 2013). *P. kessleri* also exhibits the same characteristics of tolerance to wastewater after a period of acclimation (Osundeko et al., 2014). In this study, these organisms were screened for their metal tolerance and uptake ability. *P. hussii*, *P. kessleri* and *C. luteoviridis* demonstrated significant Cd, Cu, Al and Zn tolerance compared to *C. reinhardtii* (Fig. 6.1-6.4). This result suggests that this strain’s adaptation or acclimation to wastewater could be linked to their ability to show high tolerance to metals compared to the non-adapted *C. reinhardtii*. The strains also demonstrated significant (P<0.05) increase in Cu content, presumably due to increased Cu uptake, compared to *C. reinhardtii* (Fig. 6.13A) while *P. kessleri* also showed significant (P<0.05) increase in Zn content compared to *C. reinhardtii* (Fig. 6.13B).

After comparing the metal tolerance and uptake of these wastewater adapted strains with *C. reinhardtii* it was interesting to know whether these strains could demonstrate any significant difference in metal tolerance and uptake when compared with the genetically engineered algal strains generated and described in Chapter 4 and 5. These natural strains showed a significant (P< 0.0001) increase in Zn and Cd tolerance compared to the GM strains (Fig. 6.14). Likewise, *P. kessleri* showed a significant increase in Zn content compared to all tested GM strains, while *P. hussii* and *C. luteoviridis* demonstrated significant increase compared to the AtHMA4 CT1 strain. The natural strains also demonstrated significant (P<0.05) increase in Cd content compared to the GM strains (Fig. 6.15). This significant increase in metal uptake and tolerance by the wastewater adapted natural strains is not a surprise, considering the fact that these strains have adapted mechanisms of tolerating harsh environmental conditions prevalent in wastewater (Osundeko et al., 2013; 2014), presumably over many generations. Although the mechanism involved in this enhanced removal of elements is not clear, it is believed that
the ability of these strains to tolerate highly toxic wastewater habitat is due to enhanced anti-oxidant activities within the microalgal cells, particularly linked to ascorbate peroxidase (APX) activity (Osundeko et al., 2013, 2014), which could contribute to their enhanced metal tolerance ability. Studies have shown that metals can mediate as catalysts in cellular oxidative reactions and hence could cause cellular toxicity as a result of oxidative damage (Osundeko et al., 2013). High metal concentration may cause increased reactive oxygen production (ROS) production such as the hydroxyl radical (OH) or hydrogen peroxide (H$_2$O$_2$). The over-production of these ROS could lead to oxidative stress. Oxidative stress caused by metals in microalgae could be responsible for much of the toxicity exerted on cells by metals (Ercal et al., 2001). Consequently, microalgae cells with high oxidative stress tolerance could be predicted to also tolerate metal toxicity. However, increased metal uptake is harder to explain and cannot be directly linked to oxidative stress tolerance.

One of the possible mechanisms adopted by microalgae to cope with increase in metal concentrations is the induction of heat shock proteins (HSPs) (Hall, 2002; Heckathorn et al., 2004). These protein are highly regulated under high temperature, however, other harsh environmental conditions can also induce these proteins, for example, heavy metal concentration, oxidative stress, low temperatures, high cell population density, and pathogen infection (Sorensen et al., 2003; Barua and Heckathorn, 2006). Studies have revealed that one of the ways HSPs increase tolerance to heavy metals in microalgae is by preventing membrane damage (Hall, 2002). Although HSPs have not been analysed in the wastewater adapted natural strains, these proteins might be one possible explanation for enhanced metal tolerance and uptake in these strains. An increase in carotenoid pigments and APX activity has also been linked with stress tolerance mechanisms in these algae strains (Osundeko et al., 2014). Carotenoids have been predicted to play a role in shielding against oxidative stress triggered by harsh environmental conditions (Li et al. 2008; Lemoine and Schoefs, 2010); thus high metal tolerance by the strains used in this study could be linked to the protective role of carotenoids and increased APX activity.
The characteristic of high biomass productivity exhibited by these natural strains (Osundeko et al., 2013) might also be useful for enhanced metal bioremediation and wastewater treatment. This characteristic makes these wastewater adapted strains a great tool for enhanced metal tolerance, though this may not have any direct link with enhanced metal uptake. It is interesting to know that these wastewater adapted natural strains have a longer life span than the *C. reinhardtii* strains; while the *C. reinhardtii* strains grow to stationary phase within 7 and 8 days then start to die, the natural strains grow faster and can last until 12 to 14 days at stationary phase without dying under the same growth conditions (result not shown). Again this characteristic may not have any direct effect on increased metal uptake but could enhance net metal tolerance by these strains. This study therefore establishes that engineering algae with metal transporters or metal binding proteins is not the only important factor in providing metals tolerance. Potentially combining metal transport/or metal binding protein traits into an oxidative stress tolerance strain might give better metal bioremediation activities.

### 6.3.2 Comparing metal tolerance and uptake between *Chlamydomonas reinhardtii* cell walled and cell wall-less strain

Although the cell wall composition of microalgae may vary depending on the species, generally they have the ability to bind metal ions. A typical *C. reinhardtii* cell wall lacks cellulose unlike the plant cell wall (Voigt 1988; Macfie and Welbourn, 2000). However, like other freshwater algal cells they have an abundance of glycoproteins (Voigt, 1988; Crist et al., 1994). The microalgal cell wall has been reported to possess a high metal binding characteristic which is complemented by the plasma membrane, in shielding the cells from possible metal toxicity (Sandau et al., 1996; Mehta and Guar, 2005). The cell wall is composed of polysaccharides and proteins containing functional groups that have strong binding affinity to metal ions (Crist et al., 1994; Haynes, 1980). However, when the cell walled and cell wall-less strains of *C. reinhardtii* were screened for metal tolerance there
was no significant difference in tolerance for all metals studied, possibly due to the metal concentrations used. Interestingly, the wall-less strain showed a significant (P<0.05) increase in Cu and Zn uptake compared to the walled strain (Fig 6.11). The cell wall being the first line of defence for the cell against metal toxicity helps to prevent the influx of metals into the cell therefore shielding the cell from toxicity; hence the presence of cell wall on *C. reinhardtii* has been previously shown to enhance metal tolerance (Macfie et al., 1994). Although information on metal tolerance and uptake by walled and wall-less algal strains is sparse, most studies are based on quantifying metal uptake by analysing metal adsorbed onto the cell wall (Lasat et al., 1996; Turner and Marshall, 1972). However, this does not really give adequate information on the cellular metal uptake. The cell wall-less strain had more internal metal ion accumulation than the walled strain, suggesting that fewer metals are being bound and retained by the cell wall. Cell wall-less strains might therefore be better for algal bioremediation since they have good internal metal tolerance.

The hypothesis that an increase in metal tolerance is related to an increase in cellular metal uptake was not supported in this study, as there was no significant difference in metal tolerance between the two strains. Although metal ion accumulation in the cell is not solely dependent on the presence or absence of cell wall (Macfie and Welbourn, 2000), the process of metal accumulation is a complex process influenced by several factors. This study therefore has shown that cell wall-less strains could do better in metal uptake than the walled strain.
7.0 General discussion

Toxic metal pollution has been on the increase and now it is becoming a great challenge to prevent and remove this pollution in the future, in recent years after the emergence of industrial revolution. There have been significant increases in the level of toxic metals in the environment as a result of industrial activities (Monterio et al., 2012). Although there have been strict environmental regulations set aside to check the influx of toxic metals into the environment, many chemical industries do not adhere to these regulations, particularly in some developing nations (Alloway et al., 1997; Harte et al., 1991; O’Connell et al., 2008; Monterio et al., 2012). This has created problems as a result of improper disposal of metal rich effluents into the environment which could have devastating ecosystem effects.

Although many metals are needed by organisms for growth and proper cell functioning, a few of these metals such as Cd do not play any significant role in the organism and could have a fatal effect even at low concentrations (Jjemba, 2004; Kaplan, 2004). The ‘recalcitrant’ nature of metals and their inability to degrade in the environment makes it a great concern for remediation to be successful (Harte et al., 1991). The ability of algae to sequester toxic metals makes it a potential tool to cope with the challenge of metal pollution. Understanding the mechanism of metal uptake by microalgae has been the focus of this study, which could help in the future use and engineering of microalgae for enhanced metal uptake.

Understanding how to control and enhance metal uptake processes could provide a turning point in metal bioremediation research. Hence adequate understanding of the mechanism involved in algal metal uptake could bring about innovative ways of improving efficiency of toxic metal bioremediation. Biotechnological techniques have advanced, such as the ability to genetically engineer microalgae, and there has been significant progress
in understanding components involved in metal homeostasis. There is still so much to be
done in understanding deeply the complex mechanism of metal uptake and homeostasis
in algae.

Several classes of protein have been implicated in the transport of metals (Hanikenne et
al., 2008). In the study, some of the putative transport proteins have been screened for
their metal specificity. In Chapter 3, *C. reinhardtii* MTP 1-4 which belong to the family of
CDF transport proteins were characterized by yeast heterologous expression for their
metal transport role. MTP1 was predicted to transport Zn and this was confirmed in this
study. In contrast it was also shown that MTP2-4 transports Mn. In Chapter 5, AtHMA4
screening in *C. reinhardtii* demonstrates the role of this gene in Cd and Zn transport.
Together these experiments clearly demonstrate that a better understanding of the metal
transport mechanism in microalgae can be used to genetically engineer these organisms
for increased metal uptake.

Apart from over-expressing metal transport proteins in algae for enhanced metal tolerance
and uptake, the engineering of strains to enhance phytochelatin and metallothionein
synthesis could also be considered in metal bioremediation studies (Cai et al., 1999);
however, a similar study has been done indirectly in this work by over-expressing the
AtHMA4 C-terminal tail in *C. reinhardtii* which arguably functions like a metallotheinein.
Another consideration in line with this study of metal bioremediation could be manipulating
the algal cell wall for increased synthesis of cell wall polysaccharides, hence making more
binding sites available for metal adsorption (Mehta and Guar, 2005). Genetically
manipulating algae for metal bioremediation should not stop at over-expressing the metal
transport proteins but other facets of the metal uptake mechanism such as engineering
the cell wall, plasma membrane, enhancing the synthesis of metallothioneins should be
explored for optimum results. Also other aspects of metal tolerance not related to
transport could be considered such as oxidative stress tolerance.
The intracellular localization of the transporters examined in this thesis would be another essential and interesting study to really understand how these proteins are functioning, and to allow conclusion of what would be the most appropriate genetic engineering approach in metal uptake and tolerance by microalgae. Although there have been predictions on the sub-cellular localization of some of the metal transport proteins in *C. reinhardtii*, most have not been experimentally confirmed. This therefore opens up a research of great interest in metal uptake and tolerance. Having a deeper understanding of this subject will give an insight of what happens to metals when they are sorbed by microalgae. However, it is not very clear why some microalgae strains that demonstrate high tolerance to some metals show low intracellular metal uptake (Fig 4.9 - 4.11 and Fig 4.13), studies on subcellular localization of the metal transport protein could give answers to this. In this thesis, attempts to determine the sub-cellular localization of CrMTP1 were unfortunately unsuccessful, though the presence of GFP and the MTP1 were detected in the transformed *C. reinhardtii*. The PCR result showed very faint bands of the genes. What should be done could be to increase the concentration of DNA extraction for transformation. Maybe this could increase the chance of getting the localization to work. This should be a subject of consideration in future studies.

The diversity of metal transport proteins and their broad range of metal specificity present an interesting biotechnological platform for enhancing the metal tolerance and uptake studies using microalgae. The ability of engineering specificity is important because unlike for natural strains tolerance and uptake to a specific pollutant could be increased or enhanced, which could overcome a serious problem which no natural strain could address. In contrast a strain could be engineered to tolerate multiple metals which may also be different to what is possible in nature. The development of engineered microalgae for bioremediation is not devoid of challenges. One major challenge could be whether or not genetically engineered microalgae could be suitable for commercial use, considering regulatory restrictions and the envisaged problems these engineered strains could pose to the environment if not properly handled (Flynn et al., 2013). However, the use of
genetically engineered strains should be with great caution considering environmental safety such as infection of the natural species with unwanted genes; although such risk could be prevented by having a having confined system where the metal polluted waste is treated and disposal done safely. It is also worth mentioning that the chances of these engineered strains expressing the function of the gene in question are not always fully guaranteed. Gene expression could be hampered by some unknown protein regulatory activities. Also some genes have very low level of expression when transformed into another organism, possibly as a result of silencing effects. There is also the challenge of identifying appropriate promoters. Apart from *C. reinhardtii*, other algae do not yet have fully annotated genome sequences and nuclear transformation of many algae species is very difficult. These and other unknown conditions could be a limitation to this approach. Despite these challenges, several studies have shown that algae and plants could be successfully engineered and used for enhanced metal tolerance and uptake (Siripornadulsil et al., 2002; Cai et al., 1999; Mills et al., 2010; Craciun et al., 2012; Verret et al., 2004).

Although the use of genetically engineered microalgae for metal removal is underway, natural strains isolated from metal contaminated environments have been considered in several studies and have proven to be successful in achieving the goal of metal removal from wastewater (Monteiro et al., 2012). These strains have demonstrated high level of metal tolerance and accumulation, with increase oxidative stress tolerance compared to their counter parts isolated from non-contaminated environments (Monteiro et al., 2012; Olumayowa et al., 2014). The use of natural strains for metal tolerance and uptake studied in Chapter 6 of this work demonstrates great ability of these strains to tolerate high level of metals. The outstanding growth performance of these strains (*P. hussii*, *P. kessleri* and *C. luteoviridis*) could be a great advantage in metal bioremediation. Rapid growth by these strains leading to an increase in biomass could enhance more binding sites for metal adsorption and hence increase total metal removal or bioremediation (Mehta and Guar, 2005). Although metal uptake can be determined on a per cell basis
from a laboratory perspective, for large-scale metal uptake, accumulation on a per cell basis may not really be important as the aim of the bioremediation is primarily to remove metals from the contaminated effluents. This suggests that natural strains isolated from metal contaminated sites with high biomass productivity and high metal tolerance could be preferred to genetically engineered strains.

Previous studies have shown that metal bioremediation using microalgae has been diversified, and several strategies have been employed. For example, the use of inactivated microalgal biomass (algaSORB) has been successfully used as a commercial adsorbent material for the treatment of metal contaminated wastewater and industrial effluents (Kaplan, 2004; Mehta and Guar, 2005). Although in this case it is worth mentioning that the cells used here are inactive and as such metabolic processes are not involved. Several studies have proven the use of inactivated microalgae cells for metal removal from wastewater to be successful and also advantageous considering the fact that metal toxicity to the cells will not be pronounced. This therefore means that much more metals could be removed from waste water and recovered with little or no concern about the metal toxicity to the cell as in the case with using live cells. In contrast, if a live strain could be engineered to both bind and accumulate more metals, including intracellular accumulation, and tolerate them highly, then metal removal efficiency may even be better. Additionally, living microalgae biomass cultivated in a reactor has also been used in the bioremediation of metals (Travieso et al., 2002). However, efficiency of metal removal in this case depends on the microalgal species and the particular metal that is being removed (Radway et al., 2001).

Several metal removal studies have also resorted to the use of immobilized microalgal biomass in recent times (Rangsayatorn et al., 2004; Akhtar et al., 2003). Some studies have demonstrated that immobilization technique enhances metal accumulation, while other studies have disagreed to that fact. It is argued that free cells do better in metal accumulation compared to the immobilised cells (Rangsayatorn et al., 2004). Reasons so
far could be that changes in cell structure may be the reason for inefficiency in metal uptake compared to free cells. Also, the fact that the cell surface is coated with a gel matrix following the immobilization process, such as with alginate, could result in the reduced availability of metal binding sites (Rangsayatorn et al., 2004). It is worthy to note that although the immobilisation technique has a great potential in metal bioremediation, economically this technique may not be reliable for metal bioremediation (Mehta and Guar, 2005). Although in contrast, immobilization removes the need to harvest the algae from a pond or reactor so may make the process logistically more feasible. Immobilization of the strains generated in this study would be useful to assess in future experiments.

All the strategies and techniques employed for the bioremediation metals are interesting, ranging from genetically manipulating microalgae, using the natural strains isolated from metal polluted sites and using immobilised microalgae; with the first two of these techniques being the subject of consideration in this thesis. One major consideration should be whether or not these techniques can be applied at the larger scale. Another vital point to consider is the economic implication of these techniques. However, the economic consideration of metal bioremediation using microalgae could be unpredictable as this research area is still growing and rapidly developing. Producing genetically engineered strains of algae for metal bioremediation could be quite expensive. The fact that most engineered strains could only have the capacity to remove or accumulate a few selected metals based on the metal specificity of the gene used for the transformation might also be another challenge. This is because most industrial effluent could be multi-metal contaminated.

Comparing bioremediation of metals using microalgae with conventional physico-chemical clean-up technologies shows that using algae is cost saving and minimizes site disturbance compared to the conventional techniques (Garbisu and Alkorta, 2003). Although it might be difficult to provide standard cost information for metal removal using plants and algae, in estimation Blaylock et al. (1997) proposed that phytoremediation
techniques will give 50-65% saving compared to the conventional techniques. For metal bioremediation using natural algae strains, the difference may not be much. Despite the fact that all the techniques discussed earlier could be used in metal bioremediation, when certain factors like economic feasibility, remediation sustainability, acceptability and environmental benefits are considered; the use of natural algal strains isolated from metal contaminated sites could be an outstanding choice.

7.1 Perspectives and future work

Characterization of metal transport proteins is an ongoing research area. The results shown in this thesis provide a platform for further studies in multiple directions. Though CrMTP1-4 have been heterologously characterised in yeast in Chapter 3 and CrMTP4 characterised in C. reinhardtii in Chapter 4 further characterization of CrMTP1-3 in C. reinhardtii would give a great insight into the metal transport roles of these proteins. In Chapter 4 it was predicted that CrMTP4 could be localized in the vacuole considering the cellular metal uptake results shown in this Chapter. It will be interesting to further confirm the sub-cellular localization of not only CrMTP4 but also CrMTP1-3. This will adequately explain where the metals are transported and stored by these proteins. Another study of significant interest will be through knocking down or knocking out CrMTP1-4 in C. reinhardtii and screening the mutants for their metal tolerance and uptake, to further determine the physiological role. This could also give a clear confirmation of the transport role of these proteins in C. reinhardtii.

The Zn and Cd tolerance and transport by AtHMA4 shown in Chapter 5 of this thesis further confirms the role of this gene in Zn and Cd transport. The potential of AtHMA4 transporting Cu and Mn as predicted by some of the results is worth considering for further study. The confirmation of this hypothesis could add to the number of metals that could be transported by AtHMA4. An interesting idea could be to combine the AtHMA4 C-terminal tail expression with the expression of a transport protein such as MTP or HMA in
algae for enhanced metal uptake and tolerance. In this study the subcellular localization of AtHMA4 in *C. reinhardtii* was not studied, although the metal intracellular metal uptake results hypothesise that AtHMA4 in *C. reinhardtii* could be localized in the vacuole or another intracellular compartment. Further work to verify this hypothesis could give further insight into the metal uptake mechanism and regulation by AtHMA4 when expressed in a foreign photosynthetic organism.

It appears that the natural algae strains isolated from wastewater sites demonstrates higher metal tolerance than the genetically engineered strains as shown in Chapter 6. Further studies like analysing the ROS tolerance of the natural and the genetically engineered strains could give an idea on the possible reason why the natural strains show high tolerance to metals compared to the engineered strains. The results of these studies could help arrive at a conclusion whether or not the adapted natural strains are better for metal bioremediation compared to the engineered strains. The work done in this thesis is laboratory based, and it would be of great significance to attempt this work at a large scale. This will go a long way to strengthen the work and give confidence that what have been done so far in the laboratory could be replicated in the field.
References


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