

Review

# Heavy metal detoxification in eukaryotic microalgae

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## Abstract

Microalgae are aquatic organisms possessing molecular mechanisms that allow them to discriminate non-essential heavy metals from those essential ones for their growth. The different detoxification processes executed by algae are reviewed with special emphasis on those involving the peptides metallothioneins, mainly the post transcriptionally synthesized class III metallothioneins or phytochelatins. Also, the features that make microalgae suitable organisms technologies specially to treat water that is heavily polluted with metals is discussed.

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## Contents

1. Introduction . . . . .	2
2. General characteristics of MtIII . . . . .	2
2.1. General structure . . . . .	2
2.2. Identification of PCS genes . . . . .	2
2.3. Class III metallothionein biosynthesis and regulation . . . . .	3
2.4. Sulfide ions and metallothionein function . . . . .	3
2.5. Sequestration and compartmentalization to the vacuole . . . . .	3
3. Class III metallothionein (MtIII) in algae . . . . .	4
3.1. Synthesis . . . . .	4
3.2. Physiological function and ecological importance . . . . .	4
3.3. Sulfide ions. . . . .	4
3.4. Sequestration into the vacuole . . . . .	5
3.5. Sequestration to the chloroplast and mitochondria . . . . .	5
3.6. The chain size. . . . .	5
3.7. MtIII and other related mechanisms for heavy metal detoxification . . . . .	6
3.8. Functional genomics of heavy metal stress in algae . . . . .	6
4. Gene encoded metallothioneins in algae . . . . .	6
5. Heavy metal removal by microalgae . . . . .	7
5.1. Heavy metals in waste water . . . . .	7

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5.2.	Algae-based biotechnologies for heavy metal remediation . . . . .	7
5.3.	Capability of different microalgal species to remove heavy metals . . . . .	7
6.	Conclusions . . . . .	7
	Acknowledgements . . . . .	8
	References . . . . .	8

## 1. Introduction

During evolution, aquatic and terrestrial organisms have developed diverse strategies to maintain an equilibrated relation with heavy metal ions present and available in the surrounding medium. Cells face two tasks, the first is to select those heavy metals essential for growth and exclude those that are not, and the second to keep essential ions at optimal intracellular concentrations (Cobbett and Goldsbrough, 2002).

Land plants, aquatic plants and algae have all attracted considerable attention for the capacity to eliminate heavy metal. Much of the knowledge concerning algae is based in observations of higher plants. The research reviewed here reflects this fact but stresses important discoveries relating to microalgae, for example, the evidence of the ecological importance of algal mediated chelating mechanisms in real environments (Ahner et al., 1995).

Microalgae, related eukaryotic photosynthetic organisms, and some fungi have preferentially developed the production of peptides capable to bind heavy metals. These molecules, as organometallic complexes, are further partitioned inside vacuoles to facilitate appropriate control of the cytoplasmic concentration of heavy metal ions, thus preventing or neutralizing their potential toxic effect (Cobbett and Goldsbrough, 2002). In contrast to this mechanism used by eukaryotes, prokaryotic cells employ ATP-consuming efflux of heavy metals or enzymatic change of speciation to achieve detoxification (for review see Nies, 1999).

The peptides discussed can be grouped into two categories:

1. The enzymatically synthesized short-chain polypeptides named phytochelatin (class III metallothioneins), found in higher plants, algae, and certain fungi.
2. The gene-encoded proteins; class II metallothioneins (identified in cyanobacteria, algae and higher plants), and class I metallothioneins found in most vertebrates, observed in *Neurospora* and *Agaricus bisporus* (not reported in algae) (Robinson, 1989b; Rauser, 1990; Steffens, 1990; Thiele, 1992; Gaur and Rai, 2001).

The short chain polypeptides, when first discovered, received the name of phytochelatin (PCs) because they were isolated from a higher plant (*Phyto*) and they had the capacity to bind cadmium ions (Grill et al., 1985; Steffens, 1990). Later, when class II metallothioneins were

found to be relevant in the responses of plants to heavy metals stresses, it was proposed to change the name of PCs to class III metallothioneins (Rauser, 1990).

## 2. General characteristics of MtIII

### 2.1. General structure

The general structure has been determined to be  $(\gamma\text{EC})_n$ -Gly where chain length “ $n$ ” fluctuates between 2 and 11 units (Rauser, 1990; Steffens, 1990; Cobbett and Goldsbrough, 2002). The molecular weight ranges from 2000 to 10000 DA (Steffens, 1990; Gaur and Rai, 2001).

It is important to note that the glutamic acid residues are not bond with cysteine by means of an  $\alpha$ -carboxyl group as in transcriptional aminoacids but with an  $\gamma$ -carboxyl group. In addition a number of structural variants, for example,  $(\gamma\text{EC})_n$ - $\beta$ Ala,  $(\gamma\text{EC})_n$ -Ser and  $(\gamma\text{EC})_n$ -Glu, have been indentified in other plant species (Gaur and Rai, 2001; Cobbett and Goldsbrough, 2002).

The principal structure of MtIII complexed with heavy metal ions has been difficult to obtain because of failures in crystallization. Approximations lead to propose a  $\text{Cd}(\text{S})_4$  coordination where cysteine thiolates are the main ligands (Rauser, 1990; Strasdeit et al., 1991).

The gamma bond between Glu and Cys which cannot be prepared by ribosomes, lead to the search for an enzyme-mediated path for the production of MtIII. Grill et al. (1989) demonstrated that MtIII are synthesized by the enzyme, phytochelatin synthase (PCS), which is a  $\gamma$ -glutamylcysteine dipeptidyl transpeptidase (E.C. 2.3.2.15) (Vatamaniuk et al., 2004). It catalyzes the transpeptidation of the  $\gamma$ -Glu-Cys moiety of glutathione ( $\gamma\text{ECG}$ ) onto a second  $\gamma\text{ECG}$  molecule to form  $\text{MtIII}_2$  or onto a MtIII molecule to produce an  $n + 1$  oligomer. The enzyme was described as a tetramer of MW 95000 with a  $K_m$  for glutathione of 6.7 mM (Steffens, 1990; Cobbett and Goldsbrough, 2002). The general mechanism involved is:  $[\gamma\text{Glu-Cys}]_n\text{-Gly} + [\gamma\text{Glu-Cys}]\text{-Gly} \rightarrow [\gamma\text{Glu-Cys}]_{n+1}\text{-Gly} + \text{Gly}$ .

### 2.2. Identification of PCS genes

Three research groups simultaneously isolated genes encoding for PCS activity in *Schizosaccharomyces pombe* (Ha et al., 1999), *Arabidopsis thaliana* (Vatamaniuk et al., 1999) and *Triticum aestivum* (Clemens et al., 1999). The expected gene products agreed with the PCS enzyme molecular weight previously characterized. PCS was found

to be a constitutive enzyme with no apparent gene regulated activity.

Another work reported that the *A. thaliana* genome possesses an additional PCS to *AtPCS* 1 named *AtPCS* 2, and when this new enzyme was cloned and expressed, it showed catalytic activity and its mRNA could be detected in the plant (Cazalé and Clemens, 2001). Moreover, previously described mutants lacking *AtPCS*1 were sensitive to  $\text{Cd}^{2+}$  (Howden et al., 1995b), so that the presence of a second transcript that cannot substitute *AtPCS*1 is certainly intriguing. Cazalé and Clemens (2001) have proposed that different compartmentalization for this enzyme occurs when *AtPCS* 2 is translated.

### 2.3. Class III metallothionein biosynthesis and regulation

It has been shown that MtIII synthesis could be enhanced by several metal salts, being  $\text{Cd}^{2+}$  the most potent activator, followed generally by  $\text{Pb}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$  and other heavy metals. Some metalloids, such as As and Se, also enhance MtIII production, but with lower activity than heavy metals (Grill et al., 1987). Treatments with heat, cold, ultraviolet radiation (UV), hormones, fungal cell wall, anoxia or oxidative stress do not induce MtIII synthesis (Steffens, 1990).

The fact that PCS activity in vitro tended to stop when heavy metal ions are removed and that in vivo the addition of a wide variety of heavy metal ions activates MtIII synthesis, lead to the proposal that PCS was a heavy-metal activated enzyme (Loeffler et al., 1989). However, Vatamaniuk et al. (2000) tested the affinity of PCS from *A. thaliana* (*AtPCS*) for various substrates such as glutathione, and glutathiones with S group chemically blocked. The calculated affinity constants indicate that glutathione S blocked with organic groups is a better ligand for PCS, interestingly the best ligand is a glutathione S-blocked with  $\text{Cd}^{2+}$ . They found that in absence of any heavy metal ion, PCS could prepare S-methyl-MtIII from S-methylglutathione indicating that PCS is an enzyme that not necessarily depends on heavy metal activation rather in glutathione with thiol blocked groups, although heavy metal activation remains being as important as 50%.

Studies on MtIII regulation have found that the enzymes involved in glutathione synthesis— $\gamma$ -glutamylcysteine synthetase ( $\gamma$ ECS) and glutathione synthetase (GS)—when overproduced or mutated in *Brassica* spp. produce a phenotype that is either tolerant or sensitive to heavy metal in the medium (Zhu et al., 1999a,b). When *A. thaliana* plants are genetically modified with sense or antisense  $\gamma$ ECS, either high glutathione or low glutathione plants can be produced. The low glutathione plants are sensitive to  $\text{Cd}^{2+}$  (Xiang et al., 2001). These results are in agreement with the hypothesis that glutathione is the primary peptide involved in binding heavy metals (Howe and Merchant, 1992; Vatamaniuk et al., 2000) and the substrate for MtIII synthesis (Grill et al., 1989; Howden et al., 1995a).

### 2.4. Sulfide ions and metallothionein function

Cysteine is part of the MtIII chelating core and is an activator of PCS (Oven et al., 2002), so its upstream synthesis also seems important for the production of MtIII and, maybe, the restrictive factor for the construction of new phenotypes appropriate for phytoremediation. Domínguez-Solís et al. (2001) overexpressed *O*-acetylserine(thiol)lyase in *A. thaliana*. This enzyme is responsible for the final synthesis of cysteine. They found that the plant could grow in higher concentrations of  $\text{Cd}^{2+}$  and accumulated more metal in the leaves.

Sulfide ions ( $\text{S}^{2-}$ ) are also present in metal–MtIII complexes (Steffens, 1990). This ions improves the stabilization of metal–MtIII compounds (Kneer and Zenk, 1997), in consequence, detoxification is also improved (Dameron et al., 1989).

The inclusion of sulfide ions in MtIII is the basis for the division of MtIII-complexes in two categories: low molecular weight (LMW) form, in which metal is bound to thiol groups, and high molecular weight (HMW) form in which sulfide inorganic ions ( $\text{S}^{2-}$ ) are incorporated in these complexes to form nanometer sized particles (Kneer and Zenk, 1997; Scarano and Morelli, 2003). The formation of the particles appears to be a matrix-mediated bio-mineralization process, in which the binding of the metal to  $\gamma$ -glutamyl peptides provides the matrix (Scarano and Morelli, 2003).

The origin of these inorganic sulfur ions is still unclear. Results obtained in a *S. pombe* mutant sensitive to  $\text{Cd}^{2+}$ , suggest that the protein sulfide oxidoreductase would be in charge of maintaining an adequate equilibrium of sulfide produced during a heavy metal stress (Vande and Ow, 1999).

### 2.5. Sequestration and compartmentalization to the vacuole

The metal–MtIII complex ends up in the vacuole of the cell. This was observed more than 15 years ago in the microalga *Dunaliella bioculata* (Heuillet et al., 1986), but has only been characterized in detail in the yeast *S. pombe* (Ortiz et al., 1995).

It has been demonstrated that *hmt1* gene complements a *S. pombe* mutant which is deficient in producing HMW metal–MtIII complexes and that its product—HMT1 protein—is a vacuolar transporter capable of internalizing LMW MtIII complexes in the yeast vacuole (Ortiz et al., 1992).

In the higher plant *Avena sativa*, the existence of an ATP dependent transporter of LMW Cd–MtIII complex has been demonstrated (Salt and Rauser, 1995), indicating that ATP-mediated internalization of MtIII complex is a common detoxification mechanism.

All of the findings mentioned before permit to propose the mechanisms proposed as illustrated in Fig. 1.

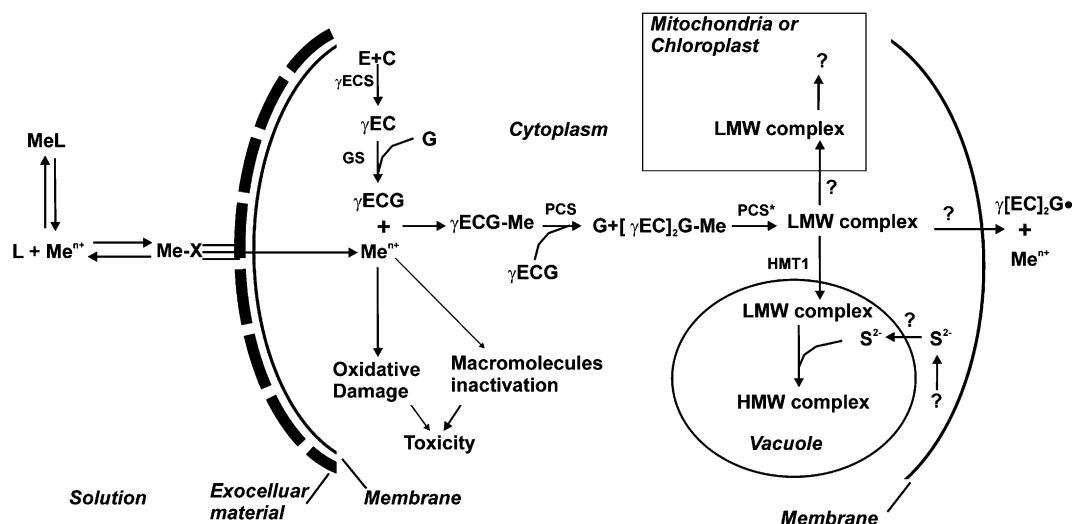


Fig. 1. General scheme of heavy metal detoxification mechanism mediated by class III metallothioneins (MtIII) in microalgae. Abbreviations: (MeL) metal complex in solution, ( $Me^{n+}$ ) free heavy metal ion, (X) biotic exocellular ligand, (E) glutamic acid, (C) cysteine, (G) glycine, ( $\gamma$ EC) gamma glutamylcysteine, ( $\gamma$ ECG) glutathione, ( $[\gamma EC]_2G$ ) metallothionein  $n = 2$ , (LMW) low molecular weight, (HMW) high molecular weight, ( $\gamma$ ECS) gamma glutamylcysteine synthetase, (GS) glutathione synthetase, (PCS) phytochelatin synthase, (HMT1) vacuolar ABC transporter. \* when this step is repeated MtIII of longer chain can be synthesized, • MtIII is disassociated when released to medium.

### 3. Class III metallothionein (MtIII) in algae

#### 3.1. Synthesis

Stokes et al. (1977) first discovered MtIII complex synthesis in the microalga *Scenedesmus acutiformis*. This was subsequently confirmed in another 11 algae belonging to six different genera and it was shown that the  $n = 2$  MtIII was the predominantly peptide synthesized in all these algae (Gekeler et al., 1988). Gaur and Rai (2001) made a tentative list of ten divisions and 24 genera of algae showing occurrence of MtIII.

MtIII biosynthesis can be induced by heavy metals such as  $Cd^{2+}$ ,  $Ag^+$ ,  $Bi^{3+}$ ,  $Pb^{2+}$ ,  $Zn^{2+}$ ,  $Cu^{2+}$ ,  $Hg^{2+}$  and  $Au^{2+}$  both in vivo and in vitro (Robinson, 1989a). Pawlik-Skowrońska et al. (2004) found MtIII accumulation when *Stichococcus bacillaris* was exposed to  $As^{3+}$ .

In concentrations of  $Cd^{2+}$ ,  $Pb^{2+}$ ,  $Ni^{2+}$ ,  $Zn^{2+}$ ,  $Co^{2+}$ ,  $Ag^+$  and  $Hg^{2+}$  occurring in non-contaminated natural waters, Ahner and Morel (1995) found induction of MtIII synthesis in *Thalassiosira weissflogii*. Knauer et al. (1998) found the same pattern in the freshwater microalga *Scenedesmus subspicatus*.

#### 3.2. Physiological function and ecological importance

Some species and ecotypes of algae can live in the presence of toxic metal concentrations that are lethal for other species or populations. MtIII clearly can have an important role in metal detoxification. Howe and Merchant (1992) reported sequestration of approximately 70% of cytosolic  $Cd^{2+}$  by MtIII in *Chlamydomonas reinhardtii*.

It has been found that MtIII synthesis is related to degree of pollution in an aquatic environment. Ahner

et al. (1994) detected a MtIII production gradient in the phytoplankton of a bay receiving anthropogenic sources of heavy metals, with higher MtIII production in zones nearer to the coast, which were likely to contained higher metal concentrations.

Torricelli et al. (2004) working with two strains of *Scenedesmus acutus* (wild type and tolerant to  $Cr^{6+}$ ) found that the constitutive concentration of cysteine was higher in the tolerant strain. When the cells were exposed to  $Cd^{2+}$ , tolerant strain showed higher levels of reduced glutathione and MtIII compared with the wild strain.

Tsuji et al. (2002) suggested that MtIII in algae could play a role not only in detoxification of heavy metal, but also in mitigation of oxidative stress. For example, Morelli and Scarano (2004) working with *Phaeodactylum tricoratum* and  $Cu^{2+}$  found that some metal-free MtIII was present in cell extracts. They made the hypothesis that this could represent an oxidized form of MtIII that had been participated to scavenge reactive oxygen species.

Add, MtIII may play an important role in essential metal ion homeostasis and sulfur metabolism in microalgae as well as in higher plants (Robinson, 1989b; Rauser, 1990; Cobbett and Goldsbrough, 2002).

#### 3.3. Sulfide ions

The role of sulfide ions in algal cultures is less known than in yeast cultures, but experimental evidence of sulfide metabolism malfunction under toxic concentrations of various metals has been observed in the marine algae, *Skeletonema costatum* and *Tetraselmis suecica*. These algae store metals as metal chelated complexes, most probable as MtIII, but also as insoluble salts in the cytoplasm (Perrein-Ettajani et al., 1999). Similar processes have been

observed in yeast mutants, lacking adequate sulfide intracellular control, when poisoned with cadmium (Vande and Ow, 2001).

Torricelli et al. (2004) working with two strains of *S. acutus* of different tolerance to  $\text{Cd}^{2+}$ , found that in response to different concentration of the metal, both strains produced increased amounts of Cys and  $\gamma$ ECG, compared with non-exposed algae. Scarano and Morelli (2003) reported that *P. tricorutum* exposed to  $\text{Cd}^{2+}$  forms Cd–MtIII complexes in which sulfide ions ( $\text{S}^{2-}$ ) can be incorporated to stabilize MtIII-coated CdS nanocrystallites. Domínguez et al. (2003) working with *C. reinhardtii* found that the presence of  $\text{Cd}^{2+}$  in the culture medium enhances the sulfate uptake rate and the components of the cysteine synthase complex within the cell such as the serine acetyltransferase and *O*-acetyl-L-serine(thiol)lyase activities.

### 3.4. Sequestration into the vacuole

It was mentioned in Section 2.5 that MtIII–metal complex inclusion in vacuoles has generated research into vacuole biochemistry hypothesis to explain tolerance and accumulation of heavy metal ions in photosynthetic organisms and fungi.

Transport of metals complexed with MtIII has only been well characterized in yeast, but in algae there are various microscopical and X-ray analyses which show that this detoxification mechanism is also occurring.

In the green algae, *D. bioculata*, electron dense materials inside the vacuoles, which contained cadmium and sulfur in a ratio between 2 and 2.4, were detected when exposed to  $100 \text{ mg l}^{-1}$  of  $\text{Cd}^{2+}$  (Heuillet et al., 1986).

The green alga *T. suecica* exposed to  $\text{Cd}^{2+}$  showed the metal accumulation in the cell wall and intracellular organelle, but also, in the vacuole as  $\text{Cd}^{2+}$  precipitation along with Ca and S it was detected (Ballan-Dufrançais et al., 1991).

In the diatom, *S. costatum*, accumulation of  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$  in vacuole of cells was detected when grown in the presence of these metals. Again a predominant element in the inclusions was sulfur in a sulfur/metal ratio of 1.5 (Nasiri et al., 1997).

### 3.5. Sequestration to the chloroplast and mitochondria

*Euglena gracilis* is a photosynthetic protist with high tolerance to  $\text{Cd}^{2+}$  and high  $\text{Cd}^{2+}$  accumulating capacity. This organism does not possess a specialized reservoir organelle such as a plant-like vacuole (Mendoza-Cózatl et al., 2004). Mendoza-Cózatl and Moreno-Sánchez (2005) working with *E. gracilis*, found that more than 60% of the accumulated  $\text{Cd}^{2+}$  resides inside the chloroplast. This was correlated with an 4.4-times increase in more thiol-compounds and sulfide, compared to a control chloroplast. In a  $\text{Cd}^{2+}$  treated chloroplast a significantly higher amount of MtIII was found, and glutathione and  $\gamma$ -EC represented the 66% of the total organic thiol content.

Avilés et al. (2003) working with  $\text{Hg}^{2+}$  pretreated heterotrophic cell of *E. gracilis* exposed to  $\text{Cd}^{2+}$ , found that 79% of the total accumulated metal was in the mitochondria. They also found a remarkable increase in the Cys and glutathione concentration in  $\text{Cd}^{2+}$  treated cells. The amount of MtIII in mitochondria was around 17% of the total MtIII found in treated cells. Mendoza-Cózatl et al. (2004) concluded that the presence of MtIII and  $\text{Cd}^{2+}$  in *Euglena* chloroplast and mitochondria, might be the result of any of the following processes:

- (1) MtIII are synthesized in the cytosol where they sequester  $\text{Cd}^{2+}$ ; the Cd–MtIII complexes are subsequently transported into the chloroplast and mitochondria.
- (2) MtIII are synthesized inside the organelle where they bind to  $\text{Cd}^{2+}$ , which are transported as free ions and then form HMW complexes.
- (3) Both processes co-exist and MtIII are synthesized in the three cellular compartments.

Interestingly, cDNAs encoding PCs, and glutathione synthetase have been reported in root mitochondria of *Brassica juncea* (Schäfer et al., 1988). The same results were found in *C. reinhardtii*, where 60% of accumulated  $\text{Cd}^{2+}$  resides inside the chloroplast, and MtIII complexes were found in this organelle (Nagel et al., 1996). Soldo et al. (2005) found that *Oocystis nephrocystioides* exposed to  $\text{Cu}^{2+}$  accumulated high concentration of the metal in the thylakoids and pyrenoid. They concluded that localization of  $\text{Cu}^{2+}$  suggests interaction of  $\text{Cu}^{2+}$  with ligands localized in the chloroplast. Alternatively,  $\text{Cu}^{2+}$  might have been transported from the cytosol to the chloroplast as  $\text{Cu}^{2+}$ –ligand complex.

### 3.6. The chain size

It has been previously demonstrated in vitro that long-chain MtIII can bind heavy metals in a stable complex (Mehra et al., 1995).

In a  $\text{Cd}^{2+}$  tolerant diatom, *P. tricorutum* ( $\text{EC}_{50} = 22.3 \text{ mg Cd l}^{-1}$ ), it was found that the size of the MtIII-chain is between  $n = 5$  and  $9$  (Torres et al., 1997).  $\text{Pb}^{2+}$  induced long chain MtIII in the same algae, but to a lesser extent than  $\text{Cd}^{2+}$  (Morelli and Scarano, 2001) and  $\text{Cu}^{2+}$  only induces MtIII  $n = 2$  (Rijstenbil and Wijnholds, 1996). In *Dunaliella tertiolecta* a marine green algae,  $n = 5$  is the major type of MtIII produced with  $\text{Zn}^{2+}$  (Hirata et al., 2001).

Torres et al. (1997) suggested that the high metal tolerance in *P. tricorutum* is not only due to an increase in MtIII production, but also to an increase in MtIII length of thiol peptides compared to other species such as *Chlorella fusca* and *S. bacillaris*. Pérez-Rama et al. (2001) concluded that *T. suecica* would be one of the most  $\text{Cd}^{2+}$  tolerant microalgae, since it is able to synthesize longer MtIII than other species.

### 3.7. MtIII and other related mechanisms for heavy metal detoxification

The MtIII mechanism viewed globally in the cell (Fig. 1) should take into account that the exclusion mechanism is also an alternative that algae possess in order to be in equilibrium with heavy metals in their environment. For example, Pistocchi et al. (2000) observed from a survey among the diatom and dinoflagellate groups that the most resistant algae to heavy metal stress were those which were capable of producing MtIII and to exocellular polysaccharides, macromolecules which provide an effective adsorbing barrier against heavy metals. This exclusion mechanism is a general feature of microalgae, not only to heavy metal stress, but also for other toxicants such as dichlorophenol as observed by Maršalek and Rojíčková (1996).

Lee et al. (1996) described in the diatom *T. weissflogii* an export system which involves MtIII. Some evidence has been found of expulsion or degradation of MtIII complexed with metal in *P. tricorutum* (Morelli and Scarano, 2001).

Rijstenbil et al. (1994) found that *Ditylum birghtwellii* (marine) and *Thalassiosira pseudonana* (riverine) under  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  stress respond with an increased activity of reactive oxygen species scavenger SH molecules. *T. pseudonana*, the more tolerant alga, maintained significant levels of antioxidative activity, MtIII production and exocellular chelating agents, but not *D. birghtwellii*.

Sexual reproduction is promoted under  $\text{Cu}^{2+}$  stress and can serve as another protective mechanism (Rijstenbil and Gerringa, 2002). Sexual reproduction as a response to severe heavy metal shock has also been observed in *Scenedesmus* spp. (Abd-EL-Monem et al., 1998). In the case of  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$ , it has been found that *Scenedesmus incrasatulus*, can respond to metal stress by expressing phenotypic plasticity that may allow these cells to survive in a hostile environment (Peña-Castro et al., 2004).

In a green alga, *C. reinhardtii*, it was found that  $\text{Hg}^{2+}$  was not chelated by MtIII, but by glutathione thus providing evidence to expand the roles which may involve glutathione not only as the  $\gamma\text{Glu-Cys}$  donor for MtIII but also as a detoxifying molecule itself, employing direct chelation (Howe and Merchant, 1992).

Members of the *Tetraselmis* genus are MtIII producers (Ahner et al., 1995; Perrein-Ettajani et al., 1999), but the species *Tetraselmis tetrathele* showed intriguing behavior under  $\text{Hg}^{2+}$  stress. It did not produce MtIII despite glutathione pool depletion, but used a novel tripeptide, Arg-Arg-Glu, with  $\text{Hg}^{2+}$  scavenging capacity and thus a possible role in detoxification (Satoh et al., 1999).

Pawlik-Skowrońska (2003) working with *Stigeoclonium tenue*, found that only the Zn-adapted ecotype exposed to high Zn and Pb was able to produce high amounts of other MtIII-related peptides, containing one additional -SH group more than MtIII.

Another mechanism by which many plants and algae respond to heavy metals is the production of proline

(Pro) (el-Enany and Issa, 2001; Bačker et al., 2004; Rai et al., 2004; Tripathi et al., 2006). Siripornadulsil et al. (2002) working with *C. reinhardtii* found that free proline acts as antioxidant in Cd-stressed cell.

### 3.8. Functional genomics of heavy metal stress in algae

There is still a lack of basic knowledge on the genetics and biochemical background of mechanisms so far reviewed. For instance, proline accumulation in response to  $\text{Cu}^{2+}$  stress observed in *Chlorella* (Wu et al., 1998) is a very interesting feature which has not been studied at this level. Other examples are the high and low affinity of heavy metals-transporters that have been kinetically characterized in green algae (Knauer et al., 1997; Sunda and Huntsman, 1998). In yeasts, however, they are already described at molecular level. Additionally, there are other mechanisms that have been described in other eukaryotic organisms, but remain unknown in algae. An example is the chaperonin activity seen in animal cells stressed by heavy metals.

With the advent of genomic projects in microalgae a way to gather basic biological information from heavy metal stress responses in microalgae, is the employment of plant functional genomic tools, such as the high-throughput screening of differential gene expression. There are several methodologies available to achieve the latter and have been reviewed elsewhere (Holtorf et al., 2002; Shrager et al., 2003).

Rubinelli et al. (2002) using mRNA differential display were able to obtain 13 cDNA sequences expressed in *C. reinhardtii* under Cd stress. Some were related to photosystem I and II maintenance, cysteine biosynthesis and Fe deficiency. More importantly, some sequences had no similarity with any reported in databases.

## 4. Gene encoded metallothioneins in algae

Animals are provided with gene-encoded peptides that can bind heavy metals and their production is tightly regulated (Thiele, 1992). The identification of sequences in plants and fungi related to class II metallothioneins (MtII) in animals expanded the scope of mechanisms available for maintaining equilibrated metal ions concentrations in these organisms (de Miranda et al., 1990), but the zones of competence of MtIII and MtII in plants is still unclear and evidence has been provided in the sense that the preference to select one of these mechanisms is related to the age of the plant, the sensitivity of the enzymes involved in glutathione synthesis to certain type of heavy metals impeding an adequate MtIII response, and the nature—essential or non-essential—of the heavy metal (Schäfer et al., 1997).

A MtII related sequence has only been identified in *Fucus vesiculosus*, a marine macroalga. Its transcripts are found to be up-regulated under  $\text{Cu}^{2+}$  stress. The protein binds  $\text{Cu}^{2+}$ , but also cadmium ions (Morris et al., 1999). In *F. vesiculosus* the MtIII response mechanism to  $\text{Cd}^{2+}$

stress has been reported, but not in response to  $\text{Cu}^{2+}$  stress (Jervis et al., 1997), so as in the case of plants, an interesting discussion about the competence of MtIII and MtII mechanisms in an organism that is able to respond with both can be expected.

## 5. Heavy metal removal by microalgae

### 5.1. Heavy metals in waste water

The anthropogenic discharge of heavy metal polluted waters into the aquatic environment, has been of world wide concern for several decades (Nriagu and Pacyna, 1988) and has led to the establishment of stronger environmental regulations concerning the release of these pollutants. Standards have been proposed on the basis of human toxicity, environmental impact, technical feasibility for reducing concentrations in effluents, and cost effective application of available technologies (Fan, 1996). Most of the currently used technologies are based on physico-chemical reactions, mainly precipitation and adsorption in ion-exchange resins. These processes face various problems such as lack of selectivity, intolerance to organic species, low efficiency in removing trace concentrations and generation of large secondary wastes with prohibitive disposal costs (Eccles, 1999).

### 5.2. Algae-based biotechnologies for heavy metal remediation

The most widely studied microbial biotreatment is based on sulfate reducing bacteria (SRB) that remove heavy metals via the production of metal-sulfide precipitates. This technology has had relative success in large-scale applications, but the main problems include long residence times (weeks), a need of continuous supply of organic substrate and large steel bioreactors (White et al., 1997).

Algae-based biotechnologies for pollution control have been used for the removal of inorganic nutrients (Hoffmann, 1998). The most common arrangements used are High Rate Algal Ponds (HRAP) (Oswald, 1988) and the patented Algal Turf Scrubber (ATS) (Craggs et al., 1996), which employs suspended biomass of common green algae (*Chlorella*, *Scenedesmus*, *Cladophora*), cyanobacteria (*Spirulina*, *Oscillatoria*, *Anabaena*) or consortia of both.

The above mentioned algal systems have been tested for heavy metal removal. Toumi et al. (2000) compared the heavy metal removal rates of traditional waste stabilization ponds (WSP) and a HRAP where both were receiving urban polluted water with trace concentrations of  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Pb}^{2+}$ . It was found that HRAP had a higher removal rate per unit volume per day, with values up to 10 times more efficient in the case of  $\text{Cu}^{2+}$ . The values obtained could have resulted from the high pH achieved as a result of algal photosynthesis that enhances metal precipitation.

Rose et al. (1998) reported a patented hybrid process which combines the advantages of HRAP and SRB. The

precipitation of heavy metal is achieved by the direct input of acid mine drainage (AMD) into a HRAP with high pH values and living biomass with adsorbing properties, then the biomass of this HRAP is recovered to be used as carbon source for SRB, the sulfide removal (via oxidation with photosynthetically produced  $\text{O}_2$ ) and polishing steps are performed in another HRAP.

ATS systems using consortia of filamentous cyanobacteria and suspended green algae have been tested for treating polluted underground waters (Adey et al., 1996). This research proved the usefulness of ATS systems for the efficient removal of heavy metals to permitted levels, and also the removal of chlorinated and aromatic organic compounds was observed. The authors hypothesized that bacteria could have aided the biodegradation of aromatic compounds. Algae degradation of these chemicals has been reported and this is a growing field of research in environmental microbiology (Semple et al., 1999).

Constructed wetlands technology for AMD attenuation may face difficulties with manganese removal and neutralizing acid pH (Mitsch and Wise, 1998). Therefore, non-agitated algal ponds have been proposed for the treatment of AMD liberated from or entering a constructed wetlands amelioration system. Phillips et al. (1995) showed that consortia of algae and cyanobacteria could effectively reduce prohibitive Mn concentrations to an environmentally safe level. In this case Mn was removed by means of biomass adsorption, high pH precipitation and immobilization.

### 5.3. Capability of different microalgal species to remove heavy metals

Various algae strains have shown appropriate properties for heavy metal removal, but most of the surveys are based in batch growth of the microalgal species.

Matsunaga et al. (1999) designed a marine screen where they were able to characterize a *Chlorella* strain capable of sustaining growth at  $11.24 \text{ mg Cd}^{2+} \text{ l}^{-1}$  and 65% removal when exposed to  $5.62 \text{ mg Cd}^{2+} \text{ l}^{-1}$ .

Travieso et al. (1999) working with *Chlorella* and *Scenedesmus* strains in batch cultures at  $20 \text{ mg Cr}^{6+} \text{ l}^{-1}$  they found removal percentages of 48% and 31%, respectively.

A high  $\text{Cd}^{2+}$  tolerant algae *P. tricornutum* ( $\text{CE}_{50} = 22.3 \text{ mg l}^{-1}$ ) which has been characterized with respect to MtIII production pattern (Torres et al., 1997) has also proved to have high removal capability (Torres et al., 1998).

*Scenedesmus* is a microalgae genus commonly used in heavy metal removal experiments. It has proven removal capacity for  $\text{U}^{6+}$  (Zhang et al., 1997),  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$  (Terry and Stone, 2002) and  $\text{Zn}^{2+}$  (Aksu et al., 1998; Travieso et al., 1999; Cañizares-Villanueva et al., 2001).

## 6. Conclusions

Algae are predominantly aquatic organisms that must be able to discriminate between essential and non-essential

heavy metal ions. In addition, they must maintain non-toxic concentrations of these ions inside their cells. In this way, two principal mechanisms have been identified, one which prevents the indiscriminate entrance of heavy metal ions into the cell, i.e., exclusion, and the other which prevents bioavailability of these toxic ions once inside the cell, i.e., the formation of complexes.

The molecules responsible for the first mechanism are extra-cellular polymers, mainly carbohydrates, and those responsible for the second are peptides derived from glutathione ( $\gamma$ ECG), the class III metallothioneins (MtIII). These MtIII peptides are a quick response of the cell to sudden and constant heavy metal stress and it is energy dependent transport to the vacuole of the cell in addition to accompanying mechanisms (like reactive oxygen species scavenging) may confer tolerance to algal phenotypes. The appearance of gene encoded class II type-metallothioneins in algae requires elucidation of the different responsibilities of both gene encoding and enzyme synthesized heavy metal binding peptides.

Macro and microalgae exhibit constitutive mechanisms for the removal of free metal ions from waters, thereby both detoxifying and remediating the water in question. Thus, making them interesting candidates as process cultures in biotreatment systems such as ATS and artificial wetlands as well as other developments.

Extensive surveys of heavy metal tolerant algae are needed in order to obtain new data, that verify and increase current knowledge of the mechanisms involved, and identify candidate enzymes for genetic manipulation, responsible for the production and transportation of MtIII.

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