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Cell wall composition affects Cd^{2+} accumulation and intracellular thiol peptides in marine red algae

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Abstract

Two red macroalgae species, *Gracilaria cornea* and *Chondrophycus poiteaui*, were evaluated for their intra and extracellular Cd^{2+} accumulation capacity, photosynthetic response and thiol peptide production. Algae were exposed for 3 and 7 days to 0.1 and 1 μg $CdCl_2$ ml^{-1} (0.89 and 8.9 μM). Intracellular accumulation of Cd^{2+} by *G. cornea* was relatively low, only comprising 20% of total metal (intracellular + extracellular). In contrast, *C. poiteaui* accumulated intracellularly close to 100% of total Cd^{2+}. In both species, metal uptake was dependent on the external Cd^{2+} concentration, metal exposure time and cell wall composition. In response to Cd^{2+} exposure, low amounts of thiol peptides were synthesized and the major difference between *G. cornea* and *C. poiteaui* was in the cell wall composition. The absence of insoluble polysaccharides in the cell wall of *C. poiteaui* suggested that this insoluble fraction might be involved in establishing an efficient barrier for the intracellular accumulation of Cd^{2+}. This is the first study in which the cell wall composition, its influence on Cd^{2+} accumulation and intracellular responses in red macroalgae are evaluated.

Keywords: Red macroalgae; Cadmium accumulation; Photosynthesis; Phytochelatins; Polysaccharides; Cell wall composition

1. Introduction

Toxic effects of metals on photosynthetic organisms include growth inhibition, photosynthetic damage and mortality, in addition to binding and blockade of essential groups in biomolecules, genotoxic damage and oxidative stress (Webster and Gadd, 1996; Mendoza-Cózatl et al., 2002; Okamoto et al., 2001). Photosynthetic organisms have developed intra and extra cellular mechanisms for metal detoxification (Clemens, 2001; Hall, 2002) and some of them, such as formation of phytochelatins (PCs) may be used as biomarkers (Ferrat et al., 2003).

The cell wall is the first barrier for metal ion uptake in higher plants and algae (Macfie and Welbourn, 2000). This extra cellular barrier has the capacity to bind metal ions in negatively charged sites. In the cell wall of marine algae, large amounts of sulfated polysaccharides are found, in contrast to freshwater and land plants (Craigie, 1990). Particularly, red algae polysaccharides have a larger capacity to bind and accumulate metals ions when compared to brown (Phaeophyta) or green (Chlorophyta) algae (Stengel et al., 2004).

One of the most widespread intracellular mechanisms for Cd^{2+} detoxification in photosynthetic organisms involves the chelation of the metal ions by peptides or proteins such as glutathione (GSH), metallothioneins (MTs) and phytochelatins (PCs) (Kotrba et al., 1999; Cobbett, 2000; Mendoza-Cózatl et al., 2005). Resistance to metals in higher plants and some green microalgae has been well documented, being attributed in most cases to the synthesis of PCs (Pawlik-Skowrońska, 2000, 2003; Torricelli et al., 2004; Kobayashi et al., 2006). However, in macroalgae, there are only a few studies on intracellular responses to metals: PC synthesis (Hu and Wu, 1998; Malea et al., 2006), conjugation with GSH (Cairrão et al., 2004) or MT identification (Morris et al., 1999).

In the present study, we analyzed the intracellular and extracellular responses to Cd^{2+} exposure in two red macroalgae with different cell wall compositions, *Gracilaria cornea* and *Chondropycus poiteaui*. Cell wall composition and thiol peptide synthesis were also analyzed in relation to the Cd^{2+} accumulation mechanisms in both species.

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2. Materials and methods

2.1. Cd^{2+} exposure

Unialgal cultures of *G. cornea* and *C. poiteaui* were established in a culture chamber under laboratory conditions at 23 °C, 80 μmol photons m^{-2} s^{-1} and 12-h light:12-h dark photoperiod with natural sea water filtered through 1.2 μm Millipore filters and 33‰ salinity. Macroalgae were grown in 1000 ml cylinders for 3–7 days in the presence of 0.1 or 1 μg $CdCl_2$ ml^{-1} in natural sea water, corresponding to 0.89 and 8.9 μM, respectively. Control cultures with no Cd^{2+} added to the culture media were also carried out.

2.2. Total and intracellular Cd^{2+} quantification

After 3 and 7 days at the indicated added $CdCl_2$ concentrations, algae were collected and rinsed with distilled water. Samples for the determination of intracellular Cd^{2+} were rinsed twice for 10 min with water containing 5 mM EDTA (pH 8) to remove metals adsorbed by the cell wall. Thereafter, the samples were freeze–dried for determination of total (water-rinsed) and intracellular accumulated Cd^{2+} (EDTA-rinsed) and dry weight. Metal content in the macroalgae was determined with a Perkin-Elmer SIMAA 6100 graphite furnace atomic absorption spectrophotometer. Samples of an *Ulva lactuca* certified reference material (BCR Reference No. 279, Commission of the European Communities) were also analyzed with every batch of samples, with an average 98% recovery with respect to the Cd^{2+} certified concentration.

2.3. Cell wall polysaccharides analysis

Macroalgal tissue was freeze–dried, pulverized, and incubated in 0.1 M sodium acetate buffer (pH 6), 5 mM EDTA, 5 mM cysteine and 0.5 mg papain. Sulfated polysaccharides were precipitated with 10% cetylpyridinium chloride (adapted from Farias et al., 2000). Soluble sulfated polysaccharides were separated from the insoluble fraction after dissolving with distilled water and further filtrating through a sintered-glass plate (4.0–5.5 μm pore size). Fractions obtained were freeze–dried and weighed (Melo et al., 2002). The sulfate content of polysaccharides was determined by a turbidimetric method (Jackson and McCandless, 1978).

2.4. Photosynthesis light–response curves

Photosynthetic responses in macroalgae exposed to Cd^{2+} were measured. Photosynthesis measurements were performed in a DW/2 chamber at 21 °C using a Clark-type oxygen electrode and the Oxygraph program (Hansatech, King's Lynn, UK). After equilibration in darkness for 3 min, respiration rate was measured as O_2 consumption. Oxygen evolution was measured during periods of 2 min at light intensities, varying from 50 to 1400 μmol photons m^{-2} s^{-1}. Maximal net photosynthetic activity (P_{max}) was calculated for each individual sample from their photosynthetic light–response curves (Henley, 1993).

2.5. HPLC analysis of intracellular thiols

Algal samples were homogenized with a mortar and pestle in liquid nitrogen and 1.5 ml of 50 mM Tris–HCl buffer (pH 8), containing 1 mM EGTA. The homogenate was centrifuged for 20 min at 4 °C and 50,000 × *g*. The supernatant was kept under reduced conditions by adding DTT (2 mM final concentration), mixed and centrifuged again for 20 min at 4 °C and 50,000 × *g*. Proteins were precipitated with perchloric acid (3% final concentration), vortexed and centrifuged as described before. The clear supernatant was filtered (using 0.22 μm pore diameter cut-off filters, Millex Millipore) and injected into a Spherisorb column (C18, reverse phase, 5μ ODS 2, 4.6 mm × 150 mm). The column was equilibrated with trifluoracetic acid (0.1%) and peptides were eluted in a 0–20% acetonitrile linear gradient at a flow rate of 1 ml min^{-1}. Post-column derivatization with Ellman's reagent was used to detect thiol-containing compounds at 412 nm, as described elsewhere (Rauser, 1991). A synthesized PC_2 standard was used to identify PC-related compounds.

2.6. Data analysis

Statistical analysis of data was carried out with STATISTICA software (Version 6, StatSoft, Inc., 2003). To determine significant differences in treatments, a non-parametric Kruskal–Wallis analysis of variance was used. The significant level was established at $\alpha = 0.05$. Data is presented as the median of four independent replicates ± one interquartile range.

3. Results

3.1. Total and intracellular Cd^{2+} quantification

Cd^{2+} uptake in *G. cornea* and *C. poiteaui* was proportional to the $CdCl_2$ concentration added to the culture medium and to the time of exposure (Table 1). Both *G. cornea* and *C. poiteaui* exhibited a high uptake when exposed for 7 days to 1 μg $CdCl_2$ ml^{-1}. *C. poiteaui* accumulated four-fold more Cd^{2+} than *G. cornea* (Table 1). However, most of the metal found in *G. cornea* was allocated extracellularly (80%). Interestingly, *C. poiteaui* accumulated most of the Cd^{2+} inside the cell (near 100% of total Cd^{2+}). Both *G. cornea* and *C. poiteaui* exhibited a higher uptake when exposed to 1 μg $CdCl_2$ ml^{-1} and after 7 days exposure.

3.2. Cell wall polysaccharides analysis

Content of soluble and insoluble polysaccharide in *G. cornea* and *C. poiteaui* is shown in Fig. 1. For *G. cornea*, polysaccharides constituted 35 ± 7% of total dry wt, 50% of which were soluble and 50% insoluble. In this species, no significant changes in the soluble/insoluble ratio were found after 3 or 7 days of exposure. In *C. poiteaui*, a lower polysaccharide content was obtained (23 ± 3% of total dry wt) when compared to *G. cornea*, but a marked difference between these species was the negligible content of the insoluble polysaccharide fraction in *C. poiteaui* (Fig. 1).

Table 1
Total and intracellular Cd^{2+} accumulation ($\mu g\ g^{-1}$ dry wt) and total thiol peptide concentrations (nmol SH mg^{-1} dry wt) in *G. cornea* and *C. poiteaui*

Exposure conditions	Total Cd^{2+}	Intracellular Cd^{2+}	Total thiol	Thiol peptides	
				PC_2	X_1
G. cornea					
Control					
3 days	0.4 ± 0.4	0.5 ± 0.5	0.7 ± 0.2		
7 days	0.4 ± 0.1	0.14 ± 0.05	0.8 ± 0.5		
0.1 $\mu g\ Cd\ ml^{-1}$					
3 days	3.4 ± 2	1.4 ± 0.7	0.7 ± 0.4		
7 days	6 ± 1	1.1 ± 0.7	0.81 ± 0.17		
1 $\mu g\ Cd\ ml^{-1}$					
3 days	14.9 ± 2.7[a]	3 ± 1.6[a]	0.8 ± 0.3	0.02 ± 0.03	
7 days	22.9 ± 2.7[a]	5 ± 1.8	1 ± 0.7	0.09 ± 0.10	0.01 ± 0.01
Kruskal–Wallis test					
3 days	$H = 9.846$	$H = 9.269$	$H = 0.356$		
	$p = 0.007$	$p = 0.020$	$p = 0.837$		
7 days	$H = 9.846$	$H = 4.718$	$H = 0.089$		
	$p = 0.007$	$p = 0.094$	$p = 0.956$		

Exposure conditions	Total Cd^{2+}	Intracellular Cd^{2+}	Total thiol	Thiol peptides		
				PC_2	X_1	X_2
C. poiteaui						
Control						
3 days	0.19 ± 0.03	0.24 ± 0.10	1.0 ± 0.9			
7 days	0.18 ± 0.03	0.33 ± 0.04	0.47 ± 0.5			
0.1 $\mu g\ Cd\ ml^{-1}$						
3 days	14 ± 4.6	14 ± 3.1	1.0 ± 0.5	0.01 ± 0.00	0.01 ± 0.01	0.02 ± 0.01
7 days	21 ± 2	23.5 ± 1.4	0.64 ± 1.44	0.01 ± 0.01	0.01 ± 0.02	0.02 ± 0.01
1 $\mu g\ Cd\ ml^{-1}$						
3 days	78.6 ± 23[a]	72 ± 25[a]	1.43 ± 0.61	0.03 ± 0.01[a]	0.02 ± 0.01	0.02 ± 0.01[a]
7 days	93.4 ± 11[a]	64 ± 12[a]	1.07 ± 0.23	0.03 ± 0.03	0.03 ± 0.02[a]	0.02 ± 0.01
Kruskal–Wallis test						
3 days	$H = 7.854$	$H = 8.910$	$H = 0.694$	$H = 6.562$	$H = 5.833$	$H = 6.562$
	$p = 0.020$	$p = 0.012$	$p = 0.707$	$p = 0.038$	$p = 0.054$	$p = 0.038$
7 days	$H = 9.846$	$H = 9.846$	$H = 2.472$	$H = 5.426$	$H = 6.325$	$H = 4.076$
	$p = 0.007$	$p = 0.007$	$p = 0.290$	$p = 0.066$	$p = 0.042$	$p = 0.130$

Median ± quartile range; $n = 4$.
[a] Significant difference with respect to control.

In the control group (no Cd^{2+} exposure), polysaccharide sulfate content was 2.6 times higher in *C. poiteaui* (20 ± 5 μg sulfate mg^{-1} polysaccharide) than in *G. cornea* (7.7 ± 2 μg sulfate mg^{-1} polysaccharide; Fig. 2). Soluble polysaccharides in *G. cornea* had a significant increase in sulfate content after 3 days exposure to 0.1 $\mu g\ CdCl_2\ ml^{-1}$; after 7 days exposure, sulfate content returned back to basal levels (Fig. 2). *C. poiteaui* did not show any significant changes in polysaccharides sulfate content after 3 or 7 days of Cd^{2+} exposure.

3.3. Photosynthetic parameters

Maximal photosynthesis (P_{max}) values in *G. cornea* and *C. poiteaui* are shown in Fig. 3. No significant differences in P_{max} were observed in *G. cornea* between treatments after 3 and 7 days of $CdCl_2$ exposure (Fig. 3). On the other hand, in *C. poiteaui*, exposure to 1 $\mu g\ CdCl_2\ ml^{-1}$ caused a considerable decrease of P_{max} after 3 days (Fig. 3). After 7 days, P_{max} values returned to control levels without significant differences with the control group.

3.4. Intracellular thiols

After 3 days exposure of *G. cornea* to 1 $\mu g\ CdCl_2\ ml^{-1}$, small production of thiol-peptides with similar HPLC retention time to phytochelatin-2 (PC_2) was detected; after 7 days exposure, a second thiol-peptide with a longer retention time was also detected (X_1), suggesting the occurrence of larger PCs in *G. cornea* (Fig. 4). However, no significant difference in total thiol content in *G. cornea* was observed (Table 1).

In *C. poiteaui*, after 3 days exposure to 0.1 or 1 $\mu g\ CdCl_2\ ml^{-1}$, three thiol peptides were found (Fig. 5). The

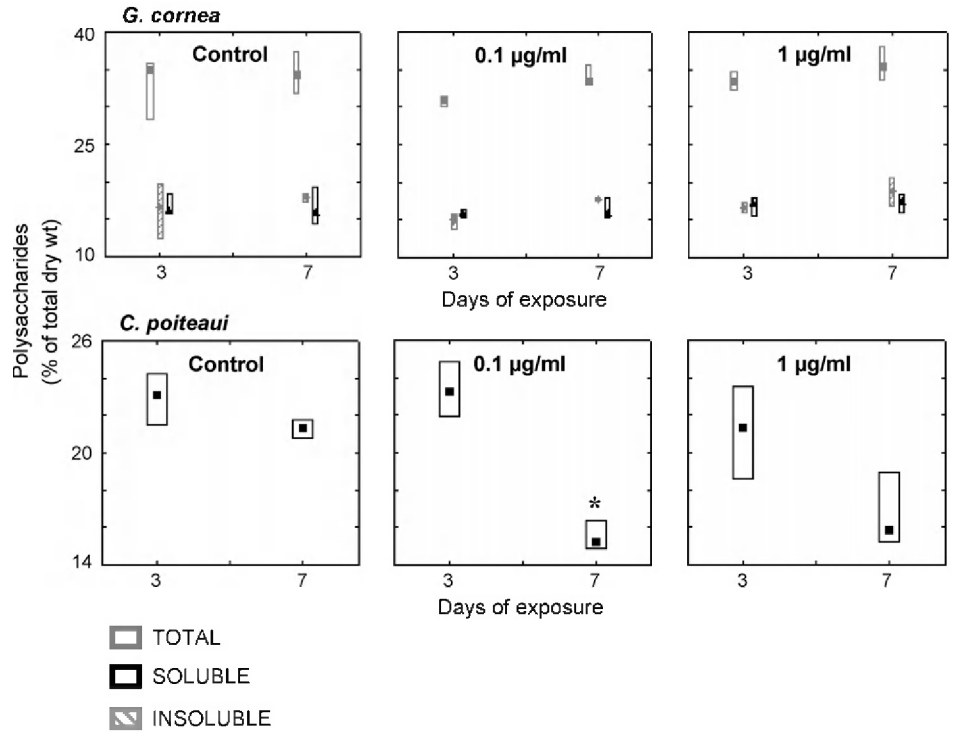

Fig. 1. Total, soluble and insoluble polysaccharides content in *G. cornea* and absence of insoluble polysaccharides in *C. poiteaui* exposed to 0, 0.1 and 1 µg $CdCl_2$ ml^{-1} in the seawater. Median ± one interquartile range ($n = 4$). *Significant differences with respect to control in *C. poiteaui* ($H = 7.848$; $p = 0.020$).

first compound exhibited a retention time similar to PC_2; two other compounds with higher retention times, possibly larger PCs, were also detected and quantified (X_1 and X_2). There was a significant increase in the content of these Cd^{2+} induced thiol-peptides after 3 and 7 days of Cd^{2+} exposure (Table 1). Although *C. poiteaui* exhibited a higher number of different thiol-compounds than *G. cornea*, the content of thiol peptides did not show significant changes between treatments.

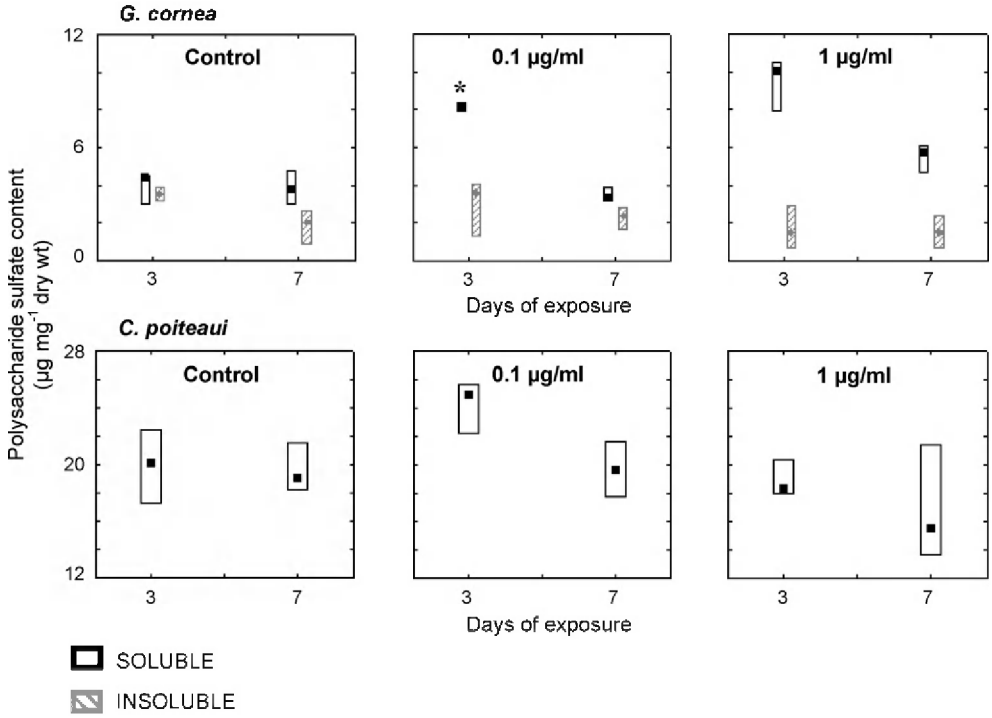

Fig. 2. Increase and recovery of sulfate content in polysaccharides of *G. cornea* and *C. poiteaui* exposed to 0, 0.1 and 1 µg $CdCl_2$ ml^{-1} in the seawater. Median ± one interquartile range ($n = 4$). *Significant differences with respect to control in *G. cornea* ($H = 6.727$; $p = 0.035$).

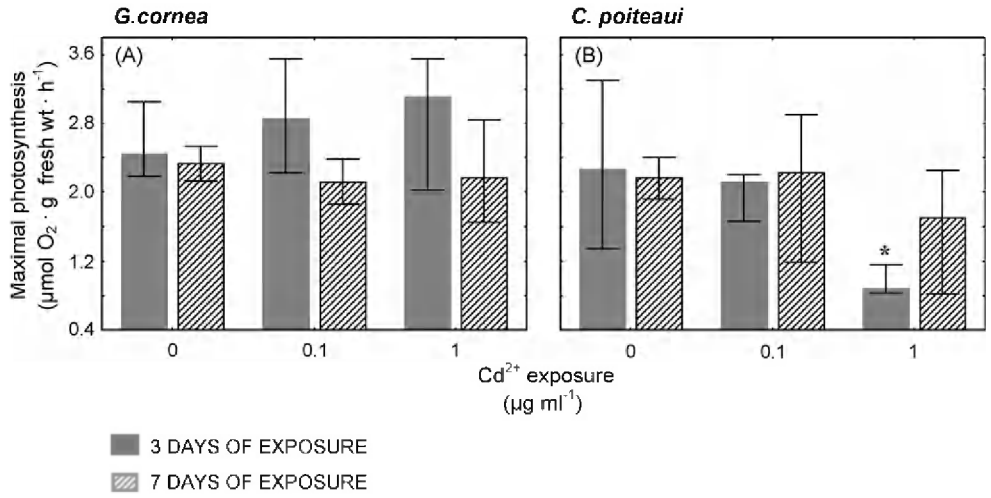

Fig. 3. Maximal photosynthesis (P_{max}) of: (A) *G. cornea* and (B) *C. poiteaui* after 3 days (grey bars) and 7 days (stripped bars) exposure to 0, 0.1 and 1 µg $CdCl_2$ ml^{-1}. Median ± one interquartile range ($n = 4$). *Significant differences with respect to control of *C. poiteaui* ($H = 7.385$; $p = 0.025$).

4. Discussion

4.1. Cd^{2+} uptake

The different capacity to accumulate Cd^{2+} by *G. cornea* and *C. poiteaui*, extra and intracellularly indicated that these species have different protection mechanisms developed against metal exposure. Apparently, the complex polysaccharides of the *G. cornea* cell wall were an efficient barrier to avoid metal toxicity, thus leading to a low intracellular metal accumulation. *G. cornea* showed a limited capacity to accumulate Cd^{2+} intracellularly, while extracellular binding correlated with the $CdCl_2$ concentration in the medium. In contrast, *C. poiteaui* predominantly accumulated Cd^{2+} intracellularly, and this process correlated with the Cd^{2+} concentration and time of exposure, although it is worth noting that the metal accumulation–$CdCl_2$ concentration relationship was not lineal. Moreover, *C. poiteaui* showed a marked cellular response to the metal exposure regarding photosynthesis and production of thiol peptides. On this regard, Wang and Dei (1999) indicated that metal uptake in *Gracilaria blodgettii* was higher at lower metal concentration in the medium.

Metal uptake is determined by the physical and chemical characteristics of the metal ion, and by the biological characteristics of the exposed organisms (Ariza et al., 1999). Maximal total Cd^{2+} accumulation in *C. poiteaui* (93.5 µg g^{-1} dry wt) and *G. cornea* (22.9 µg g^{-1} dry wt) was far below data obtained in other

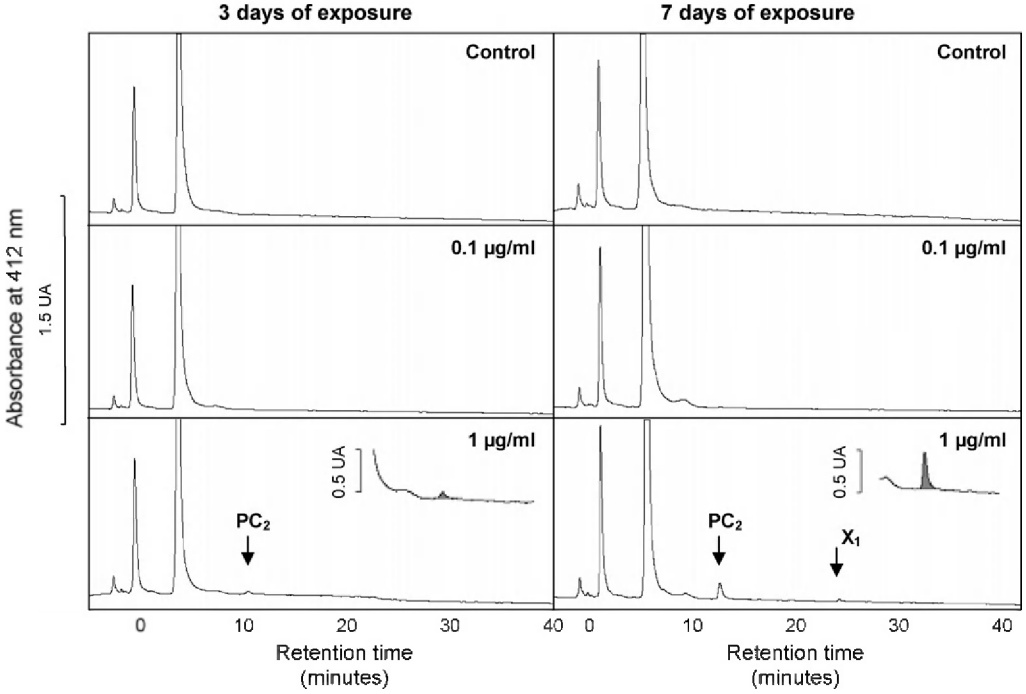

Fig. 4. Representative chromatograms of thiol peptides of *G. cornea* exposed to 0, 0.1 and 1 µg $CdCl_2$ ml^{-1} in the seawater.

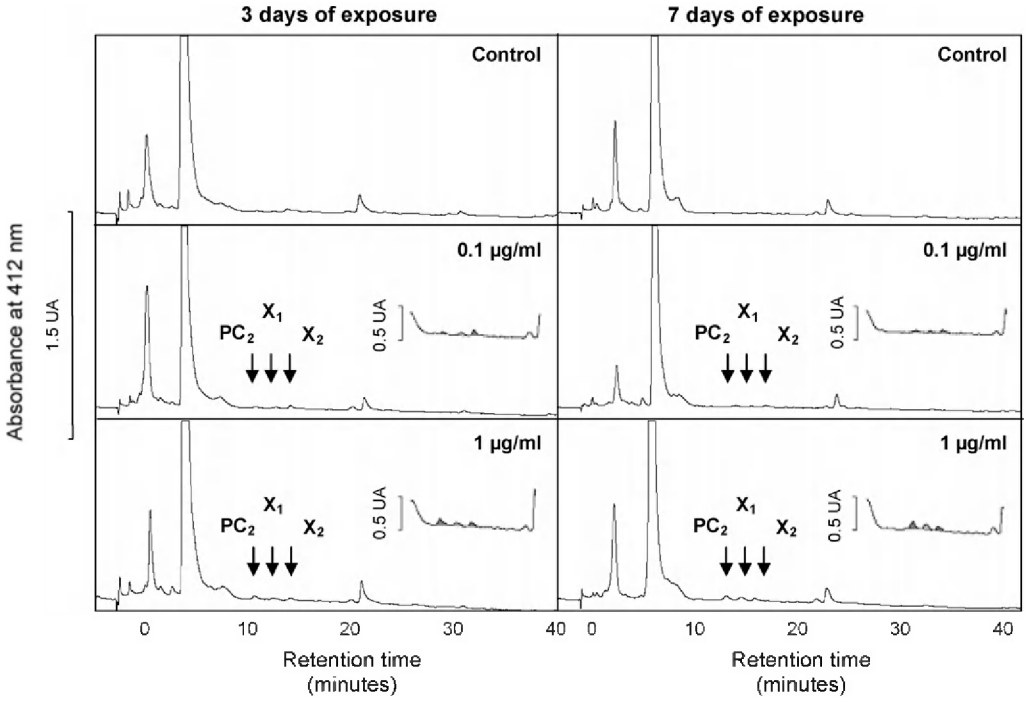

Fig. 5. Representative chromatograms of thiol peptides of *C. poiteaui* exposed to 0, 0.1 and 1 µg $CdCl_2$ ml^{-1} in the seawater.

studies with microalgae and brown macroalgae. For example, in *Chlamydomonas reinhartii*, Cd^{2+} accumulation (Kobayashi et al., 2006) was 500 and 1800-fold higher than that obtained by *C. poiteaui* and *G. cornea*, respectively, at similar added Cd^{2+} concentrations in the medium. In *Fucus serratus*, copper accumulation reached 1000 µg g^{-1} dry wt when exposed to 0.8 µM copper (Nielsen et al., 2003).

4.2. Cell wall polysaccharide and sulfur content

No evident changes in soluble or insoluble polysaccharides content were found after Cd^{2+} exposure in *G. cornea*. In contrast, soluble polysaccharides in *C. poiteaui* decreased after 7 days of exposure to both Cd^{2+} concentrations used. This response may be explained by intracellular metal accumulation and the associated perturbation of cellular functions. In *G. cornea*, its high content of cell wall insoluble polysaccharides might have facilitated the extracellular binding of Cd^{2+} and hence intracellular synthesis of the cell wall and other intracellular processes remained protected. On the other hand, absence of cell wall insoluble polysaccharides in *C. poiteaui* correlated with a negligible external Cd^{2+} binding and significant intracellular accumulation of Cd^{2+}, thus inducing alteration in cell wall synthesis and other intracellular functions.

It has not been clearly established whether cell wall composition determines metal accumulation, or it plays a protective role in microalgae. On this regard, increased polysaccharide production and low resistance, or moderate production of polysaccharides and very efficient exclusion mechanisms have been observed in phytoplankton by Pistocchi et al. (2000). In two *Chlamydomonas reinhardtii* strains, variation in the composition of the cell wall did not correlate with the different tolerance to metal toxicity (Macfie and Welbourn, 2000). A comparison of Cd^{2+} bio-absorption capacity among different macroalgae showed that alginic acid in the cell wall of brown macroalgae was more efficient to bind Cd^{2+} than polysaccharides found in red or green macroalgae (Hashim and Chu, 2004). In contrast, Stengel et al. (2004) showed that red macroalgae were more efficient to accumulate zinc than the other algal groups. The lack of insoluble polysaccharides in *C. poiteaui* (see Fig. 1) may be related to the observed high intracellular accumulation of Cd^{2+} in this species; however, more studies are necessary to determine the role of cell wall insoluble polysaccharides of red algae in Cd^{2+} accumulation.

Although sulfur containing compounds are responsible for Cd^{2+} chelation in brown algae (Raize et al., 2004), sulfate content was not related to the extracellular Cd^{2+} binding capacity between the two species used in the present study. *C. poiteaui* exhibited higher polysaccharide sulfate content; however, it did not retain Cd^{2+} in the cell wall. On the contrary, sulfate content in the soluble fraction of *G. cornea* polysaccharides increased after 3 days of Cd^{2+} exposure, returned to basal concentrations after 7 days. Seasonal sulfate content changes occurred in *G. cornea* (Freile-Pelegrin and Robledo, 1997). Increased sulfate content could be related to a general cell response to stress of multiple origins and not to a specific protective response to Cd^{2+} exposure.

4.3. Cellular response to Cd^{2+}

Accumulation of Cd^{2+} in chloroplasts and its effects on photosynthesis have been documented in microalgae (Okamoto et al., 2001; Mendoza-Cózatl et al., 2002). In *Gonyaulax polyedra*, acute exposure to Cd^{2+} generated oxidative stress in the chloro-

plasts, whereas under chronic exposure the antioxidant system was able to protect (Okamoto et al., 2001). The decrease of P_{max} in *C. poiteaui* after 3 days of exposure and its recover after 7 days may reflect the activation of the antioxidant system. Analysis of the degree of oxidative stress and antioxidant enzymes activity are necessary to determine the effect of metal exposure on photosynthetic responses. On this issue, P_{max} recovery in *C. poiteaui* after 7 days of Cd^{2+} exposure may be related to metal detoxification mechanisms. This suggestion is consistent with the synthesis of thiol-peptides beginning after 3 days and increasing after 7 days of Cd^{2+} exposure. As PC synthesis was induced at low, almost negligible levels, it may be proposed that PC synthesis is not a significant part of the Cd^{2+} detoxification mechanisms in this species. Higher induction of PC synthesis in microalgae exposed to Cd^{2+} concentrations similar to those used in this study has been previously reported (Mendoza-Cózatl et al., 2005; Kobayashi et al., 2006), as well as after exposure to other metals such as zinc and lead (Pawlik-Skowrońska, 2000, 2003; Tsuji et al., 2003).

GSH is the major reservoir (up to 90%) of non-proteic sulfur in the cell, and is the substrate for PC synthesis. Consumption of GSH by PC synthesis induces an increase in the rate of GSH synthesis to restore basal levels. For that reason, depending on the GSH demand rate, GSH levels may increase or decrease after Cd^{2+} exposure (reviewed by Mendoza-Cózatl et al., 2005). In our results, total thiol content did not change with Cd^{2+} exposure; in other words, there was no significant PC synthesis. In the two marine macroalgae analyzed in the present study, Cd^{2+} exposure caused a low PC synthesis without GSH depletion.

No significant synthesis of PCs in *G. cornea* and *C. poiteaui* was achieved at the low intracellular Cd^{2+} concentration accumulated. In higher plants and some yeast, the number of Cd^{2+} ions bound by the thiol group is variable (<1–4), and depends on the thiol-compound chemical nature (i.e. GSH, PCs, etc.) (Zenk, 1996). In the present study, the thiol group content/intracellular Cd^{2+} ratio in *C. poiteaui* was 2:1, which suffices to chelate all intracellular Cd^{2+} without PC synthesis, although Cd^{2+} should not be fully inactivated as a thiol/Cd^{2+} ratio of 4 is required. This response suggests that red macroalgae have detoxification mechanisms that involve active plasma membrane metal uptake, and an efficient intracellular metal binding by compounds other than PCs, perhaps smaller thiol-compounds. A low PC induction has also been observed in the green macroalgae *Enteromorpha* spp. (Malea et al., 2006). Other possible intracellular mechanisms different to PCs may also be responsible for metal detoxification in some marine macroalgae. Non-protein reduced sulfur like cysteine and glutathione might be the major thiol compounds involved in the detoxification of all intracellular Cd^{2+} in this macroalgae whereas PCs may play only a minor role.

5. Conclusions

Soluble and insoluble polysaccharides present in the cell wall of *G. cornea* bound most of the Cd^{2+} extracellularly. The absence of insoluble polysaccharides in the cell wall of *C. poiteaui* was the major difference when compared to the *G. cornea* cell wall. In *C. poiteaui*, most of the Cd^{2+} was accumulated intracellularly, which however did not induce a significant PC synthesis and therefore other detoxification mechanisms may be involved. The recovery of the photosynthetic performance of *C. poiteaui* after 7 days of Cd^{2+} exposure suggested the activation of intracellular detoxification mechanisms. However, more studies on oxidative stress and antioxidant enzymes activity are required to determine the effect of metal exposure on these physiological responses.

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