Cadmium toxicity on the freshwater microalga *Chlamydomonas moewusii* Gerloff: Biosynthesis of thiol compounds

Cristina Suárez, Enrique Torres, Mónica Pérez-Rama, Concepción Herrero, Julio Abalde

Laboratorio de Microbiología, Facultad de Ciencias, Universidade da Coruña, 15008. A Coruña, Spain

Environmental Toxicology and Chemistry, Volume 29, Issue 9, September 2010
Submitted 16 February 2009; Returned for Revision 16 February 2010; Accepted 19 March 2010

Abstract

Cadmium (Cd) toxicity and production of different thiols (phytochelatins, glutathione, γ-Glu-Cys and cysteine) were studied in the microalga *Chlamydomonas moewusii* exposed to different concentrations of this metal (1, 2, 4, 6, 8, and 10 mg/L) for 96 h. The inhibitory effect of Cd on growth was demonstrated. The value of EC50 (metal concentration which reduces the population growth to 50% of the control) obtained for this microalga was estimated at 4.1 ± 0.8 mg/L of Cd after 96 h of exposure. The amount of thiol compounds synthesized by *C. moewusii* changed with Cd concentration. Cysteine concentrations were significantly higher compared to those of γ-Glu-Cys and glutathione in all the Cd concentrations assayed. The amino acid cysteine reached its higher levels in those cultures in which a decrease in the

concentration of phytochelatins (PCs) was observed. Both cysteine and glutathione concentrations showed significant differences along the Cd concentrations assayed, while the amount of γ-Glu-Cys detected remained stable. The PCs detected were of two, three, and four subunits. The level of PC_2 was higher than that of PC_3 and PC_4. PC_4 was detected only in the cultures exposed to the Cd concentrations of 1 and 2 mg/L, in which the synthesis of phytochelatins was higher. A rapid increase in the production of PC_2 and PC_3 was observed up to a Cd concentration of 2 mg/L, after which their levels began to decrease. Phytochelatins were not detected in cultures without Cd (controls) and in those exposed to the maximum Cd concentration (10 mg/L), in which cell growth was completely inhibited

Keywords

Thiols; Phytochelatins; Cadmium; Chlamydomonas moewusii; Toxicity

INTRODUCTION

Widespread pollution by heavy metals that is generated by human activities has serious effects on human health and the environment. Heavy metals are among the most toxic pollutants that affect the survival of aquatic organisms. This is because, unlike other pollutants (weed-killers, pesticides), they remain in the environment without degradation. Therefore, they accumulate in sediments and can be released slowly into the water, exercising their toxicity even in the long term. In addition, metals accumulate along the food chain, causing a serious threat to human health [1]. In particular, Cd is one of the most dangerous heavy metals present in ecosystems, which causes their deterioration. It affects, in various ways, all organisms in the environment, including microalgae. The damaging effects of Cd on microalgae are of particular significance considering the essential role played by these photoautotrophic microorganisms as primary producers and initial link in the food chain.

The presence of Cd in the environment is increasing due to its use in industrial processes [2]; it is a common industrial pollutant. Because of this increase, microalgae have developed different tolerance mechanisms to try to counteract its toxicity. One of the most common responses to Cd stress by microalgae is the production of thiol compounds [3]. Phytochelatins (PCs: small, thiol-containing peptides) are one of the most important thiol compounds synthesized against this stress. Phytochelatins have the amino acid structure $(\gamma\text{-Glu-Cys})_n$ – Gly, where *n* ranges from 2 to 11. They are structurally related to the tripeptide glutathione (GSH; γ-Glu-Cys-Gly). Several physiological, biochemical, and genetic studies have confirmed that GSH (or related

compounds) is the substratum to the PCs biosynthesis [4, 5]. Tripeptide glutathione acts as the base unit of PCs. At the same time, the amino acid cysteine is the main precursor of GSH via synthesis of γ-Glu-Cys. The biosynthesis of cysteine is required under conditions of metal stress to allow the increase in the biosynthesis of GSH and PCs [3]. Different reports have shown that cysteine is also involved in metal detoxification [6].

Phytochelatins are synthesized enzymatically from the GSH in a sequential two-step reaction, by the enzyme γ-glutamyl-cysteinyl-dipeptidyl transpeptidase (phytochelatin synthase) [7]. This enzyme catalyzes the reaction by which the oligomer grows by the addition of γ-glutamyl-cysteinyl-dipeptidyl (γ-Glu-Cys) molecules to the phytochelatin peptide chain. This dipeptide is derived from GSH. Genes for this enzyme have been isolated from *Schizosaccharomyces pombe*, wheat, and *Arabidopsis thaliana* [8].

The main property of PCs is their ability to chelate metals intracellularly, playing an important role in metal tolerance [9]. The role of PCs in Cd^{2+}detoxification has been established by showing that *S. pombe* and *A. thaliana*mutants, lacking synthesis of PCs or GSH, are Cd^{2+} sensitive [10]. Compared to metallothioneins (MTs), PCs offer many advantages that are due to their unique structural characteristics. Their metal-binding capacity is higher than that of MTs; in addition, PCs can incorporate high levels of inorganic sulfide, which results in very significant increases in the Cd^{2+}-binding capacity of these peptides [11]. The complex formed between PCs and Cd^{2+} occurs through coordination with the sulfhydryl group of the cysteine, forming thiolate-metal complexes. This complex chelates the metal in the cytosol of the cell, and therefore it is inactivated. This avoids the inhibitory effects of the metal on active catalytic sites or on structural proteins [5]. Once Cd^{2+} is chelated by PCs, these complexes are carried toward the vacuolar system. This metal-chelating ability is an argument in favor of their role in metal tolerance [12].

Cadmium is the main inductor of PC synthesis, although synthesis of PCs has also been reported in algae exposed to high concentrations of Cu [13] and Zn [14]. However, Tripathi et al. [15] observed that the level of total thiols in *Scenedesmus* sp. substantially declined during both short- and long-term exposure to Cu and Zn, and therefore phytochelatins seemed not to be of much importance in conferring tolerance to *Scenedesmus* sp. against metal-induced oxidative stress. In the same way, Tsuji et al. [14] observed that phytochelatins enhanced the tolerance of *Dunaliella tertiolecta* against metal-induced oxidative stress but only when the GSH level was not depleted in the cells.

The objectives of the present study were to examine Cd toxicity on the freshwater microalga *Chlamydomonas moewusii* Gerloff and to study the capacity of this microalga to synthesize PCs in presence of this metal. Besides the study of PCs synthesis, the

concentrations of cysteine, γ-Glu-Cys, and GSH were also measured in response to different Cd^{2+} concentrations.

Chlamydomonas moewusii Gerloff is an unicellular green freshwater microalga from the family *Chlamydomonaceae*. This microalga has been used in toxicological studies with herbicides [16] and is an important organism model for cytological studies. Therefore, it is also interesting to know its response to metals, especially to Cd^{2+}, which is one of the most toxic heavy metals and inducer of PCs. The role of PCs (and related molecules) in tolerance to Cd is discussed in this paper.

MATERIALS AND METHODS

Test organism and culture condition

The microalga species chosen for this study was *Chlamydomonas moewusii*Gerloff (*Chlamydomonadaceae*). This strain was obtained from the CCAP Culture Collection of Algae and Protozoa of Freshwater Ecology Institute (Cumbria, UK) (strain CCAP 11/5B). It was grown and maintained in autoclaved (121°C, 20 min) Bristol medium [17]. Microalgal cultures were incubated at a temperature of 18 ± 1°C, illuminated with 68.25 μmol photon/(m^2 s), with a dark:light cycle of 12:12 h and continuously aerated with a constant flux of 10 L/min. All experiments were performed with this culture medium.

Chemicals and reagents

All chemicals were of the highest purity available. Cd chloride 2 ½-hydrate ($CdCl_2$. 2 ½ H_2O), orthophosphoric acid (H_3PO_4), boric acid (H_3BO_3), hydrochloric acid (HCl), sodium hydroxide (NaOH), monobromobimane ($C_{10}H_{11}N_2O_2Br$), sodium borohydride ($NaBH_4$), diethylenetriaminetetraacetic acid anhydride ($C_{14}H_{19}N_3O_8$), ethylenediaminetetraacetic acid (EDTA), lugol and Tris (hydroxymethyl) amino methane (TRIS) were purchased from Sigma. High-performance liquid chromatography-grade methanol was purchased from Scharlau Chemie. Filters were obtained from Millipore (Millipore Ibérica). The different reagents and buffers were prepared with Milli-Q® water obtained from a Milli Q Plus system (Millipore Ibérica).

Cadmium stock

A stock solution of Cd was prepared by dilution of cadmium chloride 2 ½ - hydrate in Milli-Q water to a final concentration of 1 g/L of Cd^{2+}. This solution was then filtered through a 0.22 μm Millipore filter.

Experiment design and metal treatment

The test cultures were grown in sterilized 1000 ml Pyrex glass bottles. For the experiments, appropriated volumes (1, 2, 4, 6, 8 and 10 ml) of the Cd^{2+} stock solution were added to a final volume of 1 L of culture medium to obtain Cd concentrations of 1, 2, 4, 6, 8, and 10 mg/L, respectively. Inocula for all experiments were taken from a culture in logarithmic phase. Each inoculum was added to each culture medium, containing each one of the different Cd concentrations assayed, yielding an initial cell density of 40×10^4 cells/ml. Control cultures without Cd^{2+} were also included. All cultures were performed in triplicate and were kept for 96 h.

Growth measurement

Growth of microalgal cultures was measured daily by counting culture aliquots in a Neubauer (Blaubrand) hemocytometer chamber after fixation with lugol. The degree of growth inhibition by Cd in *C. moewusii* cells was measured during the 96 h of culture. Total cells present in a known volume of culture were counted and then cell density was calculated. The Cd^{2+}concentration that reduces the population growth to 50% of the control growth level (EC50) was calculated using a log dose-response curve. Analysis of the data was conducted by applying a nonlinear regression to log metal concentration versus the difference, respect to the control, of the final cell densities obtained after 96 h of culture. These analyses were performed using SigmaPlot 9.01 software package (Systat Software). The EC50 was calculated from the regression equation. Logistic equation of four parameters was chosen for its goodness-of-fit [18].

The growth rate (μ), expressed as 1/d, was obtained from the following equation:

$$\mu = [\ln(N_t) - \ln(N_0)]/[\ln 2(t - t_0)]$$

where t_0 and t are the initial and final time of the exponential growth period, both expressed as days, and N_0 and Nt are the number of cells/ml at those times.

Characterization and quantification of cadmium-induced thiols

Chlamydomonas moewusii cells were harvested by centrifugation (1500 *g* for 10 min) from a certain volume of each culture after 96 h of growth, and stored at −20°C until the analysis. Thiol peptides were then extracted from the frozen samples and derivatized with monobromobimane (mBrB) that specifically labels sulfhydryl-containing compounds [19].

A capillary electrophoresis technique was used to measure GSH, γ-Glu-Cys, the amino acid cysteine, and PCs previously derivatized with the monobromobimane [19]. Using this technique, all the thiols mentioned previously could be analyzed in the same run. The equipment used was an $HP^{3D}CE$ (Capillary Electrophoresis System; Agilent Technologies) and the electropherograms were analyzed with the Agilent ChemStation Software (Agilent Technologies). Standards of PCs, cysteine, γ-Glu-Cys, and GSH, derivatized in the same way as the biological samples, were used to identify the different peaks.

Data analysis

Data were expressed as means ± standard error. All data were analyzed using the statistical program SPSS 12.0 (SPSS Iberica, Madrid, Spain). The results for the effect of Cd^{2+} on microalgal growth and for the different thiols production were tested by one-way analysis of variance (ANOVA). Finally, data were compared with the Dunnett test. The α-level for significant differences was set at $p < 0.05$.

RESULTS

Cadmium effect

Chlamydomonas moewusii

Cultures of *C. moewusii* exposed to different Cd^{2+} concentrations (1, 2, 4, 6, 8 and 10 mg/L) showed significant differences in their growth (Fig. 1) as a result of the toxic effect of the metal. The ANOVA test ($p < 0.05$) revealed a significant effect of Cd^{2+} on the growth of *C. moewusii* after 96 h of exposure. This inhibitory effect was directly proportional to metal concentration. Even at 24 h of culture, differences in the growth among the cultures were detected. Those differences became larger as time of exposure increased. This observation proved the inhibitory effect of Cd^{2+} in the growth of this microalga. Cultures with Cd^{2+} concentrations of 6 and 8 mg Cd/L hardly grew, and in those with the highest Cd^{2+} concentration assayed (10 mg/L), total inhibition of growth was observed. The cellular density of the cultures with higher Cd^{2+} concentrations did not vary throughout the 96 h of culture.

The statistical analysis, applying the Dunnett test to the values of the cellular density reached after 96 h of exposure, showed that the toxic effect of the different Cd^{2+} concentrations assayed on *C. moewusii* growth could be expressed as $0<1<2<4<6<8 = 10$ (Fig. 1). From this sequence, it is possible to deduce that the lowest observed effect concentration (LOEC) obtained in this study was 1 mg Cd/L. The no

observed effect concentration (NOEC) was not observed in this assay, or it can be considered the control culture (0 mg Cd/L).

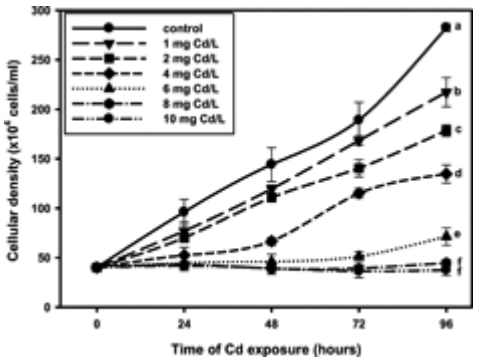

Figure 1. Growth of *C.moewusii* cells with the Cd^{2+} concentrations assayed. Each data point represents mean ± standard error of 3 replicates. Data points marked with the same letter are not significantly different.

The data of the growth rate obtained for the cultures exposed to the different Cd^{2+} concentrations assayed, also proved the toxic effect of Cd^{2+} on the growth of this microalga, since this value decreased as the Cd^{2+} concentration increased (Table 1). The ANOVA test ($p < 0.05$) applied to the growth rates confirmed this significant effect of the metal, and the data obtained using the Dunnett test showed that this toxic effect could be expressed as 0>1>2>4>6>8 = 10 (Table 1). These results, both for the cellular density at 96 h of growth and for the growth rates, showed that the toxic effect of Cd^{2+} was evident between each pair of Cd^{2+} concentrations, except those with 8 and 10 mg Cd/L, in which the total inhibition of the growth was observed.

Table 1. Growth rate ± standard error (1/d) of *Chlamydomonas moewusii*cultures exposed to different Cd^{2+} concentrations after 96 h. Data marked with asterisks are not significantly different.

Cadmium concentration (mg/L)						
control	1	2	4	6	8	10
0.70 ± 0.01	0.61 ± 0.02	0.54 ± 0.01	0.44 ± 0.02	0.21 ± 0.04	0.04 ± 0.004 *	0

Dose–response curves are useful to describe a toxic action in relation with the conditions of the experiment. Thus, Figure 2 shows the dose–response curve for the growth of *C. moewusii*, and the EC50 was calculated from the regression equation applied. The toxic effect of Cd^{2+} can be also measured directly through this parameter. The value of the median effective concentration (EC50) for *C. moewusii* was estimated at 4.1 ± 0.8 mg/L of Cd^{2+}after 96 h of exposure to the metal.

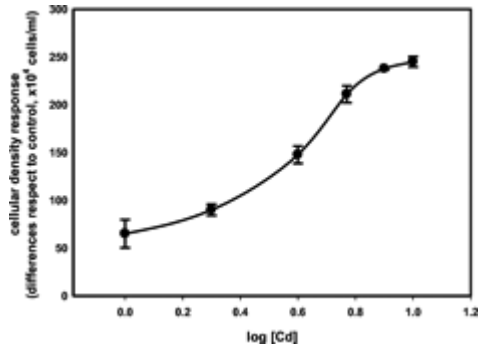

Figure 2. Dose – response curve for the growth of *C. moewusii* after 96 h of exposure to the different Cd^{2+} concentrations. Each data point represents mean ± standard error of 3 replicates.

Glutathione, cysteine, and γ-Glu-Cys production

Figure 3 shows the concentration of cysteine, γ-Glu-Cys and glutathione reached in the different cultures of *C. moewusii* exposed to different Cd^{2+}concentrations and in cultures without metal addition after 96 h of exposure. The synthesis of the amino acid cysteine depended on the Cd^{2+} concentration added to the medium. A great increase in the cysteine concentration was observed after 96 h of culture in *C. moewusii* cells exposed to Cd^{2+}. The amount of cysteine in all the Cd^{2+} concentrations assayed was significantly higher than that of glutathione and γ-Glu-Cys ($p < 0.05$). A significant increase was observed in cysteine content of the cells as Cd^{2+} concentration increased up to 4 mg/L. The amount of cysteine remained with no significant variations ($p > 0.05$) among the Cd^{2+} concentrations of 4 and 8 mg/L, in which it reached its highest value. At 10 mg/L of Cd^{2+}, an important reduction in the cysteine concentration took place, reaching only a value of 68 ± 8.79 amol/cell, even lower than in the control cultures. Thus, the ANOVA test showed significant differences in the cysteine levels between the different Cd^{2+} concentrations ($p < 0.05$). In addition, the statistical analysis using the Dunnett test showed that these differences in the cysteine production are significant among all the Cd^{2+} concentrations assayed except those with 4, 6 and 8 mg Cd/L, in which the higher levels of cysteine reached were not significantly different.

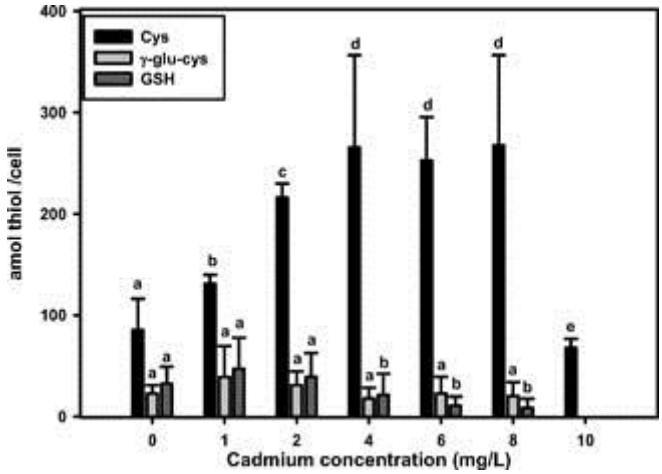

Figure 3. Production of glutathione (GSH), γ-glutamylcysteine (γ-EC), and the amino acid cysteine in *Chlamydomonas moewusii* cells in response to different Cd^{2+} concentrations expressed as amol thiol/cell (where $a = 10^{-18}$), after 96 h of exposure. Data bars represent mean ± standard error of 3 replicates. Data bars marked with the same letter are not significantly different.

The concentrations of GSH and γ-Glu-Cys observed were very low compared with the values of the amino acid cysteine achieved. The statistical analysis for GSH, using the ANOVA test, showed that the synthesis of this low-molecular-weight thiol presented significant changes along the Cd^{2+} concentrations tested ($p < 0.05$), except for that of 10 mg Cd/L, in which both GSH and γ-Glu-Cys were not detected. A slight decrease in the GSH levels along the Cd^{2+}concentrations assayed is also observed in the Figure 3. The amount of GSH decreased as the cysteine level increased. The highest amount of GSH was detected in the culture with 1 mg/L of Cd^{2+}, with a value of 47.1 ± 30.27 amol/cell. However, γ-Glu-Cys did not present significant differences in its levels along the Cd^{2+} concentrations assayed ($p > 0.05$). Nevertheless, the values of cysteine observed in all the Cd^{2+} concentrations assayed were much higher than those of GSH and γ-Glu-Cys.

Cadmium effect on PCs production

In the present study, the synthesis of PCs of two, three and four subunits ($(\gamma\text{-EC})_2G$, $(\gamma\text{-EC})_3G$ and $(\gamma\text{-EC})_4G$)) in response to the exposure of *C. moewusii* to Cd^{2+} was detected (Fig. 4). The synthesis of these PCs was not detected in the control cultures with the applied technique.

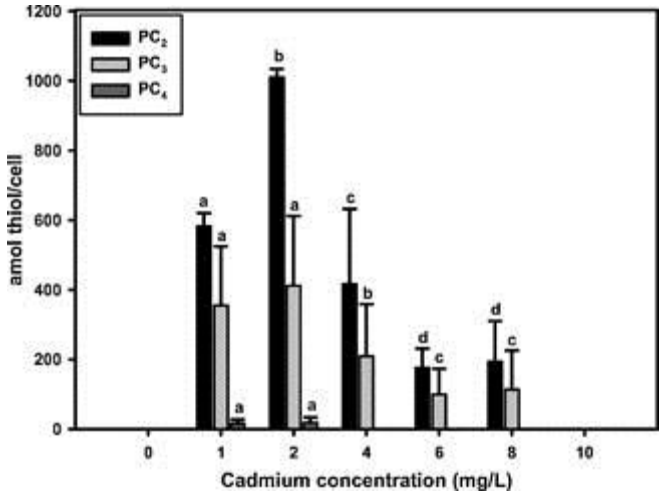

Figure 4. Production of phytochelatins in *Chlamydomonas moewusii* cells in response to different Cd^{2+} concentrations, expressed as amol thiol/cell (where $a = 10^{-18}$), after 96 h of exposure. Data bars represent mean ± standard error of 3 replicates. Data bars marked with the same letter are not significantly different.

In relation to PC_4, it was only detected in the cultures in which the highest synthesis of PCs occurred (1 and 2 mg/L of Cd^{2+}), but the concentration of PC_4observed was much lower than that of PC_2 and PC_3.

The concentration of PC_2 was much higher than that of PC_3 in all the cultures where they were detected, but both PCs followed the same evolution along the different Cd^{2+} concentrations assayed. Their levels increased until the cultures exposed to 2 mg/L of Cd^{2+} in which they reached their maximum values (1010 ± 23.44 amol/cell and 411 ± 170 amol/cell, respectively). In higher Cd^{2+} concentrations, the levels of PCs decreased significantly until the concentration of 8 mg Cd^{2+}/L. They were not detected in the cultures exposed to 10 mg/L of Cd^{2+}, in which the growth cell was completely inhibited.

DISCUSSION

It is well recognized that Cd is classified as one of the most dangerous metals, with a severe toxicity for both aquatic and land organisms 2. This is the reason why this metal has been chosen for this study. *Chlamydomonas moewusii* is frequently studied in laboratory bioassays, but few data on Cd toxicity and tolerance are available for this species. With respect to cell growth, *C. moewusii* is a species with a high tolerance to Cd, whose total inhibition is achieved only at concentrations higher than 8 mg Cd^{2+}/L.

Growth inhibition is a common symptom of Cd toxicity, but many studies have shown that there is a wide range of sensitivity to Cd^{2+} among microalgal species. These

differences occur even within species of the same genus. This happens, for example, between *Tetraselmis gracilis* with an EC50 value of 1.8 mg Cd/L [20] and *Tetraselmis suecica* with an EC50 of 7.9 mg Cd/L after 144 h of exposure [21]. Marine microalgae are among the organisms that tolerate higher Cd^{2+} concentrations. The growth of *Chlorococcum* sp. and *Tetraselmis gracilis* is inhibited at concentrations of 2.5 to 3 and 5 mg/L of Cd^{2+}, respectively [20], which also indicates a high resistance to Cd^{2+}. Folgar et al. [22] demonstrated that *Dunaliella salina* is one of the other most tolerant species to this metal. It tolerated a Cd concentration as high as 40 mg Cd^{2+}/L along 96 h of culture, and the EC50 value obtained was 48.9 mg Cd/L. However, a study was performed by Visviki and Rachlin [23] in which the EC50 obtained for the same microalga was much lower, although the culture conditions were completely different.

Contrary to these highly tolerant microalgal species, other species are very sensitive to Cd^{2+}, such as *Asterionella formosa*, whose growth is inhibited at concentrations of 0.01 μg/L of this metal [24]. The EC50 value obtained for the microalga *Isochrysis galbana* after 96 h of exposure was 0.74 mg Cd/L [25].

Many studies have demonstrated that freshwater microalgae are generally more sensitive to Cd^{2+} than marine microalgae. Thus, for *Chlorella sp*., growth is inhibited with a concentration of 1.12 mg/L of Cd^{2+}, with a LD50 of 0.34 mg/L at 72 h of culture [26]. For *Scenedesmus vacuolatus*, the growth rate was still optimal at a Cd^{2+} concentration of 0.12 μg/L [27]. However, Mosulen et al. [28] found that Cd^{2+} ions are toxic for the growth of*Chlamydomonasreinhardtii* at a concentration of 33.7 mg/L after 96 h of culture. Likewise, Kobayashi et al. [29] also observed that *C. reinhardtii* CC125 cells could grow in medium containing up to 14 mg Cd/L.

The EC50 obtained in the present study for *C. moewusii* was estimated at 4.1 ± 0.8 mg/L of Cd^{2+} after 96 h of exposure. Because of this high value, it was necessary to expose the microalga to high Cd^{2+} concentrations to reach complete inhibition of growth. It was exposed to a maximum Cd^{2+}concentration of 10 mg/L, although growth inhibition was already observed at a Cd^{2+} concentration of 8 mg/L (Fig. 1).

Although this EC50 value is lower than that obtained for *C. reinhardtii* [28, 29], when it is compared with the values obtained for other freshwater microalgae, it can be concluded that *C. moewusii* is a microalga very tolerant to Cd^{2+} ions. Thus, considering the efficiency for metal removal that microalgal cells have [30], this microalga could be used as a good organism to remove high amounts of this metal from the environment. Based on these growth traits, it is suggested that *C. moewusii* has a potential in bioremediation of Cd-contaminated waters, because tolerance to metal toxicity is a crucial feature for accumulators.

The natural concentration of Cd in the environment is very low (usually less than 0.5 µg/L in clean water), but industrial development requires an increase in its use, which leads to higher levels of this metal in aquatic environments, causing their deterioration 2. Microalgae are directly exposed to this pollution and therefore must develop tolerance mechanisms. Several mechanisms are known that allow these microorganisms to develop protection against high levels of Cd, being the intracellular ones, some of the most studied. Within this group, phytochelatins stand out. Several studies have demonstrated that Cd^{2+} is one of the most powerful inductors of PC biosynthesis. Because of this, PCs have been the mechanism of defense most investigated with regard to Cd^{2+} tolerance [4, 31].

For example, Alvarez-Legorreta et al. [32] observed that in the seagrass*Thalassia testudinum*, all tissues tested (green blades, live sheaths and root/rhizomes) experienced an increase in thiol-containing compounds after 144 h, as a response to Cd^{2+} exposure, with PC_2 reaching the highest values. This PC synthesis showed a significant positive correlation with respect to Cd^{2+} exposure. Like the results obtained by these authors, the present study also found that the PC synthesized in higher amount by *C. moewusii* was PC_2. Synthesized PCs bind Cd^{2+} to form the complex PC - Cd^{2+}. In this complex, Cd^{2+} is bound to –SH groups of these molecules [26]. There are many studies that show that PCs are the main intracellular metal chelators induced by Cd^{2+}treatments; in fact, it was observed that PC complexes sequestered more than 70% of the total Cd^{2+} found in cells treated with this metal [33]. It was also observed that *T. suecica* synthesized PCs in cultures with a Cd^{2+}concentration of 6 mg/L, and about 87% of the accumulated Cd^{2+} was bound by these molecules [34]. Furthermore, it has been proposed that the synthesis of short chain PCs is the result of a relatively low-level heavy metal exposure, whereas the synthesis of long chain PCs presumably is caused by exposure to high heavy metal levels [35]. In the present study with *C. moewusii*, a significant increase of PCs of 2 and 3 subunits was also observed, reaching high levels in the cultures exposed to Cd^{2+} until a concentration of 8 mg Cd/L. Phytochelatins of four subunits were also detected, but it was only observed in the cultures exposed to 1 and 2 mg Cd/L. The concentration reached was very low. It is noteworthy that the amounts of PC_2 and PC_3increased until the Cd^{2+} concentration approached the obtained EC50 value. At higher Cd^{2+} concentrations, the amounts of these PCs began to decrease, which reveals their role as a mechanism of tolerance to Cd^{2+} developed by this microalga. In fact, at these metal concentrations, the growth of *C. moewusii* showed an important reduction (Table 1).

With regard to other microalgae species belonging to the genus*Chlamydomonas*, Kobayashi et al. [29] observed that PCs were obviously detected in *C. reinhardtii* 3 h after the addition of 5.6 to 11.25 mg Cd/L, whereas they were not found in the presence

of 1.12 mg Cd/L even after 6 h. However, in the present study an increase in the levels of PCs was detected, even in *C. moewusii* cells exposed to 1 mg Cd/L, although this observation was obtained 96 h after the addition of Cd to culture medium. Kahoko et al. [36] even confirmed different subtypes of PCs, from PC_2 to PC_6, in cultures with only 0.0067 mg Cd/L and after 96 h of exposure, in the strains of *C. acidophila* KT-1, *C. acidophila* DVB238 and *C. reinhardtii* C-9. They observed that *C. acidophila* KT-1 and *C. reinhardtii* C-9 mainly synthesized PC_2, which is also the most synthesized PC by *C. moewusii* in the present study (Fig. 4). These authors assumed that PCs play an important role in Cd tolerance in *Chlamydomonas* strains. This statement is also confirmed by the results obtained with *C. moewusii*.

Phytochelatins are rich in cysteine, and therefore their biosynthesis requires large amounts of this amino acid. Thus, tolerant microalgae should have a higher capacity to absorb, reduce and assimilate sulfate. Pérez-Rama et al. 3found that in the marine microalga *Tetraselmis suecica,* the amino acid cysteine exceeded in all cultures the concentration reached in controls. The amount reached by this amino acid was higher at the higher Cd^{2+}concentrations (15 and 30 mg/L), after 7 d of exposure. In the present study, the cysteine concentration also increased with the exposure of *C. moewusii* to Cd^{2+}, reaching the highest level at 8 mg Cd/L. Thus, as it happened with PCs, this amino acid also increased its level until the Cd^{2+} concentration that is near the EC50 value. But contrary to the PCs, the amount of cysteine remains stable at higher Cd^{2+} concentrations. Therefore, it acts as an important mechanism of defense to the metal in those cultures of 4, 6 and 8 mg Cd/L, in which a decrease in the levels of PCs was observed. This observation could suggest that sulfate reduction is a very robust pathway able to support high cysteine demand, even in circumstances, as those of the present study with*C. moewusii*, in which the cysteine increase significantly when the microalga is exposed to high Cd^{2+} concentrations (Fig. 3). Quite the opposite of these results, Mendoza-Cózatl et al. [37] found no significant differences in the cysteine content in live sheaths of plants among the different Cd^{2+} exposure treatments.

Kaplan et al. [26] observed that, as a response to GSH addition to cultures of*Chorella* sp. cells exposed to Cd^{2+}, there was an increase in the abundance of PCs. There are previous reports showing that GSH attenuates the inhibitory effect of Cd^{2+} in tomato cell cultures [38]. Nishikawa et al. [39] observed, using different strains of *Chlamydomonas acidophila*, that the PC content did not correlate closely with the level of Cd accumulation. They suggest that maintenance of a high GSH level seems to be more important for Cd accumulation. In the present study with *C. moewusii*, after 96 h of culture, significant differences were observed in the GSH levels among the Cd^{2+}concentrations assayed. GSH reached its maximum value at the Cd^{2+}concentration of 1 mg/L (below the EC50) and it begins to decrease gradually as the metal

concentration increases, until 8 mg Cd/L. In the highest Cd^{2+} concentration (10 mg/L), where the toxicity of Cd is very evident, it was not detected (Fig. 3). Furthermore, these levels are correlated with those obtained for the PCs (Fig. 4). The levels of PCs are not only dependent on the activity of PC synthase but also on the amount of the substrate GSH. In *C. moewusii*, GSH levels did not decrease while PC levels increased. This behavior of the GSH is precisely due to that it acts as the main precursor molecule for the biosynthesis of the PCs, whose levels also decreased as Cd^{2+} concentration increased (Fig. 4).

Finally, the other important molecule in the synthetic pathway of PCs is γ-Glu-Cys. The γ-Glu-Cys content observed did not change significantly among the Cd^{2+} concentrations assayed and with respect to the control cultures (Fig. 4). The amount of γ-Glu-Cys remained quite stable and was very low, compared with that of cysteine. This seems to indicate that the activity of γ-Glu-Cys synthetase did not increase greatly because of Cd exposure. Similar observations were also obtained in the marine microalga *T. suecica* [21].

CONCLUSIONS

In the present study, it can be concluded that Cd has a toxic effect on the growth of *Chlamydomonas moewusii*. However, in the group of freshwater microalgae, *C. moewusii* is one of the most tolerant species to Cd, since it possesses a high value of EC50 (4.1 ± 0.8 mg Cd/L at 96 h of exposure). The complete inhibition of its growth was reached in the cultures exposed to a high Cd^{2+} concentration (8 mg/L). There was an important synthesis of the amino acid cysteine, dependent on the Cd^{2+} concentration assayed, reaching its maximum level in the cultures exposed to 4 and 8 mg Cd/L. This amino acid remained with higher levels in those cultures in which a decrease in the concentration of PCs was observed. The concentrations of GSH and γ-Glu-Cys observed were much lower than that reached by the cysteine. A significant decrease in the GSH levels was observed as the Cd^{2+} concentration increased from 1 mg/L, whereas γ-Glu-Cys did not show significant variations among the Cd^{2+} concentrations tested.

The production of PCs of 2 and 3 subunits was observed in the cultures exposed to Cd^{2+} until a concentration of 8 mg/L of this metal. The maximum value of both PCs was reached in the cultures exposed to 2 mg Cd/L, being much higher the concentration observed of PC_2. Phytochelatins of 4 subunits were also detected, but only in the cultures exposed to 1 and 2 mg Cd/L, and their concentrations were very low in comparison with PC_2 and PC_3. PCs were not detected in the cultures without Cd^{2+} (controls).

Because of its high tolerance to Cd and its ability to synthesize PCs in the presence of the metal, it could be concluded that *C. moewusii* is a good candidate, among freshwater microalgae, to be used in bioremediation processes. This would be useful information in practical applications such as phytoremediation of waters polluted by Cd, and it could contribute to developing new techniques to deal with the problem of metal toxicity in the environment.

Acknowledgements

Cristina Suárez Díaz holds a Fellowship Award from the Xunta de Galicia.

REFERENCES

1. De la Noue J, De Paw N. 1998. The potential of microalgal biotechnology: A review of production and uses of microalgae. *Biotechnol Adv* **6**: 725–770.
2. Mislin H, Ravera O. 1986. Cadmium in the Environment. Vol 50. Birkhäuser Verlag Basel, Basel, Switzerland.
3. Pérez-Rama M, Torres E, Abalde J. 2006. Composition and production of thiol constituents induced by cadmium in the marine microalga Tetraselmis suecica.*Environ Toxicol Chem* **25**: 128–136.
4. Cobbett C. 2000. Phytochelatins and their role in heavy metal detoxification. *Plant Physiol* **123**: 825–833.
5. Zenk MH. 1996. Heavy metal detoxification in higher plants - a review. *Gene* **179**:21–30.
6. Ahner BA, Wei L, Oleson JR, Nori O. 2002. Glutathione and other low-molecular-weight thiols in marine phytoplankton under metal stress. *Mar Ecol Prog Ser* **232**: 93–103.
7. Tsuji N, Nishikori S, Iwabe O, Shiraki K, Miyasaka H, Takagi M, Hirata K, Miyamoto K.2004. Characterzation of phytochelatin synthase-like protein encoded by alr0975 from a prokaryote, Nostoc sp. PCC7120. *Biochem Biophys Res Commun* **315**: 751–755.
8. Ha S-B, Smith AP, Howden R, Dietrich WM, Bugg S. 1999. Phytochelatin synthase genes from Arabidopsis and the yeast, Schizosaccharomyces pombe. *Plant Cell* **11**:1153–1164.
9. Mason AZ, Jenkins KD. 1995. Metal detoxification in aquatic organisms. In TessierA,TurnerDR, eds Metal Speciation and Bioavailability in Aquatic Systems. John Wiley & Sons, Chichester, UK, pp 479–608.
10. Mutoh N, Hayashi Y. 1988. Isolation of mutants of Schizosaccharomyces pombe.*Biochem Biophys Res Commun* **151**: 32–39.
11. Mehra RK, Mulchandani P, Hunter TC. 1994. Role of CdS quantum crystallites in cadmium resistance in Candida Glabrata. *Biochem Biophys Res Commun* **200**:1193–1200.

12. Grill E, Winnacker E-L, Zenk MH. 1985. Phytochelatins: the principal heavy-metal complexing peptides of higher plants. *Science* **230**: 674–676.

13. Nagalakshmi N, Prasad MNV. 2001. Responses of glutathione cycle enzymes and glutathione metabolism to copper stress in Scenedesmus bijugatus. *Plant Sci* **160**:291–299.

14. Tsuji N, Hirayanagi N, Okada M, Miyasaka H, Hirata K, Zenk MH, Miyamoto K. 2002.Enhancement of tolerance to heavy metals and oxidative stress in Dunaliella tertiolecta by Zn-induced phytochelatin synthesis. *Biochem Biophys Res Commun* **293**:653–659.

15. Tripathi BN, Gaur JP. 2006. Physiological behavior of Scenedesmus sp. during exposure to elevated levels of Cu and Zn and after withdrawal of metal stress.*Protoplasma* **229**: 1–9.

16. Prado R, García R, Rioboo C, Herrero C, Abalde J, Cid A. 2009. Comparison of the sensivity of different toxicity test endpoints in a microalga exposed to the herbicide paraquat. *Environ Int* **35**: 240–247.

17. 17Brown TE, Richardson FL, Vaughn ML. 1967. Development of red pigmentation in *Chlorococcum wimmeri* (Chlorophyta: Chlorococcales). *Phycologia* **6**: 167–184.

18. Organization for Economic Cooperation and Development. 2006. Freshwater Algae and Cyanobacteria, Growth Inhibition Test. OECD Guidelines for the Testing of Chemicals. Vol 201. Geneva, Switzerland.

19. Pérez-Rama M, Torres E, Abalde J. 2005. Capillary zone electrophoresis for analysis of phytochelatins and other thiol peptides in complex biological samples derivatized with monobromobimane. *Electrophoresis* **26**: 610–620.

20. Okamoto OK, Asana CS, AIdar E, Colepicolo P. 1996. Effects of cadmium on growth and superoxide dismutase activity of the marine microalga Tetraselmis gracilis(Prasinophyceae). *J Phycol* **32**: 74–79.

21. Pérez-Rama M, Abalde J, Herrero C, Torres E. 2002. Cadmium removal by living cells of the marine microalga Tetraselmis suecica. *Bioresour Technol* **84**: 265–270.

22. Folgar S, Torres E, Pérez-Rama M, Cid A, Herrero C, Abalde J. 2009. Dunaliella salina as a marine microalga highly tolerant to but a poor remover of cadmium. *J Hazard Mater***165**: 486–493.

23. Visviki I, Rachlin D. 1994. Acute and chronic effect exposure of Dunaliella salina and Chlamydomonas bullosa to copper and cadmium: effects on ultraestructure. *Arch Environ Contam Toxicol* **26**: 154–162.

24. Rachlin JW, Warkentine BE, Jensen TE. 1982. The growth responses of Chorella saccharophila, Navicula inserta and Nitzschia clorsterium to selected concentrations of cadmium. *Bull Torrey Bot Club* **109**: 129–135.

25. Yap CK, Isamail A, Omar H, Tan SG. 2004. Toxicities and tolerances of Cd, Cu, Pb and Zn in a primary producer (Isochrysis galbana) and in a primary consumer (Perna viridis). *Environ Int* **29**: 1097–1104.

26. Kaplan D, Heimer YM, Abeliovich A, Goldsbrough PB. 1995. Cadmium toxicity and resistance in Chlorella sp. *Plant Science* **109**: 129–137.

27. Le Faucheur S, Behra R, Sigg L. 2005. Phytochelatin induction, cadmium accumulation, and algal sensitivity to free cadmium ion in Scenedesmus vacuolatus. *Environ Toxicol Chem* **24**: 1731–1737.

28. Mosulen S, Dominguez MJ, Vigara J, Vilchez C, Guiraum A, Vega JM. 2003. Metal toxicity in Chlamydomonas reinhardtii. Effect on sulfate and nitrate assimilation. *Biomol Eng***20**: 199–203.

29. Kobayashi I, Fujiwara S, Saegusa H, Inouhe M, Matsumoto H, Tsuzuki M. 2005. Relief of arsenate toxicity by Cd-stimulated phytochelatin synthesis in the green alga Chlamydomonas reinhardtii. *Mar Biotechnol* **8**: 94–101.

30. Tüzün I, Bayramoğlu G, Yalçin E, Başaran G, Çelik G, Arica Y. 2005. Equilibrium and kinetic studies on biosorption of Hg(II), Cd(II) and Pb(II) ions onto microalgae Chlamydomonas reinhardtii. *J Environ Manage* **77**: 85–92.

31. Rauser WE. 1990. Phytochelatins. *Annu Rev Biochem* **59**: 61–86.

32. Alvarez-Lagorreta T, Mendoza-Cózatl DG, Moreno-Sánchez R, Gold-Bouchot G. 2008.Thiol peptides induction in the seagrass Thalassia testudinum (Banks ex König) in response to cadmium exposure. *Aquat Toxicol* **86**: 12–19.

33. Hu S, Lau KWK, Wu M. 2001. Cadmium sequestration in Chlamydomonas reinhardtii. *Plant Sci* **161**: 987–996.

34. Pérez-Rama M, Herrero C, Abalde J, Torres E. 2001. Class III metallothioneins in response to cadmium toxicity in the marine microalga Tetraselmis suecica (Kylin) Butch. *Environ Toxicol Chem* **20**: 2061–2066.

35. Torres E, Cid A, Herrero C, Abalde J. 1998. Removal of cadmium ions by the marine diatom Phaeodactylum tricornutum Bohlin accumulation and long-term kinetics of uptake. *Biores Technol* **63**: 213–220.

36. Kahoko N, Onodera A, Tominaga N. 2006. Phytochelatins do not correlate with the level of Cd accumulation in Chlamydomonas spp. *Chemosphere* **63**: 1553–1559.

37. Mendoza-Cózatl DG, Loza-Tavera H, Hernández-Navarro A, Moreno-Sánchez R. 2005.Sulfur assimilation and gluthatione metabolism under cadmium stress in yeast, protists and plants. *FEMS Microbiol Rev* **29**: 653–671.

38. Mendum SC, Gupta SC, Goldsbrough P. 1990. Effect of gluthatione on phytochelatin synthesis in tomato cells. *Plant Physiol* **93**: 484–488.

39. Nishikawa K, Onodera A, Tominaga N. 2005. Phytochelatins do not correlate with the level of Cd accumulation in Chlamydomonas spp. *Chemosphere* **63**: 1553–1559.