

# Composition and production of thiol constituents induced by cadmium in the marine microalga *Tetraselmis suecica*

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## **Abstract**

Time course of intracellular levels of different thiols (phytochelatin, desglycyl-phytochelatin, glutathione,  $\gamma$ -glutamylcysteine [ $\gamma$ -Glu-Cys], and cysteine) were studied in the microalga *Tetraselmis suecica* exposed to different cadmium concentrations for 8 d. The cadmium concentrations assayed were 3, 7.9, 15, and 30 mg/L. Contents of thiol compounds synthesized by this microalga changed with cadmium concentration and with time of exposure. Cysteine concentrations increased significantly compared with those of  $\gamma$ -Glu-Cys and glutathione in cultures containing 7.9, 15, and 30 mg/L. The

increase in the amount of glutathione was significant only in cells exposed to the higher cadmium concentrations (15 and 30 mg/L). Nevertheless, in all the cadmium concentrations assayed,  $\gamma$ -Glu-Cys levels were constant. A rapid increase in phytochelatins was observed with increased cadmium concentration during the first days of culture. The type of phytochelatins (number of subunits) also was dependent on the concentration of cadmium. The rate of  $(\gamma$ -Glu-Cys)<sub>3</sub>-Gly accumulation was higher than those of other types of phytochelatins throughout the culture in cells exposed to 3, 15, and 30 mg/L. However, in cultures treated with 7.9 mg/L,  $(\gamma$ -Glu-Cys)<sub>4</sub>-Gly increased substantially after 8 d of culture, exceeding the amount of  $(\gamma$ -Glu-Cys)<sub>3</sub>-Gly. Cultures exposed to the higher cadmium concentrations (15 and 30 mg/L) showed a decrease in phytochelatins after 4 and 3 d of culture, respectively, whereas an increase in cysteine and glutathione occurred at the same time. A rapid decrease in phytochelatins also was measured when cells were placed into cadmium-free medium. This decrease is consistent with a degradation of the phytochelatins.

### **Keywords**

Thiols; Phytochelatins; Cysteine; Cadmium; Microalga

### **INTRODUCTION**

Different environmental studies have shown that coastal areas often are affected by cadmium pollution. This pollution causes loss of biological diversity and increased bioaccumulation and magnification of cadmium in the food chain. The survival capacity of different aquatic organisms, including phytoplankton, depends on the presence of specific responses that prevent the toxicity of this metal.

Microalgae have developed different resistance mechanisms, such as adsorption of metal ions to cell wall components, excretion of organic compounds forming extracellular complexes with the metal ions, and production of metal ion chelators either in the cytosol or at intracellular level [1].

Phytochelatins (small, thiol-containing peptides) are assumed to act as intracellular chelators [2,3]. These molecules are oligo- and polypeptides with the amino acid structure ( $\gamma$ -glutamylcysteine [ $\gamma$ -Glu-Cys])*n*-Gly; also abbreviated as  $\gamma$ -

EC<sub>n</sub>G). Phytochelatins with *n* ranging from 2 to 11 have been described [4,5]. Although glycine is the most common terminal amino acid, glutamic acid [6], serine [7], and β-alanine [8] have been found instead of glycine in several species. Moreover, the terminal amino acid does not appear in desglycyl-phytochelatins [9]. In these molecules, cadmium is chelated throughout coordination with the thiol group of cysteine.

Phytochelatins are synthesized enzymatically from the tripeptide glutathione (GSH) by a γ-glutamyl-cysteinyl-dipeptidyl transpeptidase (phytochelatin synthase) [10,11]. The enzyme cleaves the terminal glycine from one GSH and catalyzes the transfer of the γ-glutamyl-cysteinyl-dipeptidyl to the amino terminus of another GSH that acts as acceptor to form the *n* = 2 oligomer; hence, the chain grows by binding additional γ-Glu-Cys moieties to the phytochelatin peptide chain.

Previous synthesis of GSH, carried out in two steps involving two enzymes, is necessary for the synthesis of phytochelatins. In the first step, γ-glutamylcysteine (γ-Glu-Cys) synthetase combines the amino acids glutamic acid and cysteine to form γ-Glu-Cys. Later, the enzyme GSH synthetase catalyzes the reaction between γ-Glu-Cys and the amino acid glycine to form GSH [4].

Therefore, GSH is the base unit of phytochelatins, and at the same time, cysteine is the main precursor of GSH via synthesis of γ-Glu-Cys. Cysteine biosynthesis is required under conditions of metal stress to allow increased biosynthesis of GSH and phytochelatins. Moreover, different reports have shown that cysteine also is involved in metal detoxification [12–14].

Once cadmium is chelated by phytochelatins, these complexes are carried toward the vacuolar system. Nassiri et al. [15,16] observed that the cadmium accumulated in the vacuoles of *Tetraselmis suecica* and *Skeletonema costatum* showed good correlation between cadmium, sulfur, and nitrogen. This suggests that phytochelatin–cadmium complexes can be sequestered in the lysosomal or vacuolar system.

Interest regarding the involvement of physiological responses in monitoring studies has been increasing. To understand the tolerance mechanism developed by phytochelatins, it is essential to know the dynamics of the synthesis and the stability of these molecules and their precursors. Few reports on the evolution of phytochelatins and their precursors, both with time of exposure and with different doses of cadmium in

microalgal cells, have been printed [3,17]. Thus, the goal of the present study was to examine the time course of synthesis, magnitude of response, and degree of polymerization of phytochelatins by *T. suecica* (a typical marine microalga) exposed to different cadmium concentrations. The intracellular concentrations of GSH,  $\gamma$ -Glu-Cys, and cysteine also were measured in response to metal stress. In the present study, a sensitive capillary electrophoresis technique was used to analyze phytochelatins and related thiols. This technique allows the quantification and characterization not only of phytochelatins but also of cysteine,  $\gamma$ -Glu-Cys, GSH, and other thiol peptides, such as desglycyl-phytochelatins, simultaneously (i.e., in the same run) [18]. Samples were derivatized previously with monobromobimane, a chromogenic compound that labels thiol groups and provides selectivity in the detection process.

Persistence of thiols in cultures (previously exposed to cadmium) transferred to a medium without metal also was monitored.

## **MATERIALS AND METHODS**

### **Chemicals and reagents**

All chemicals were of the highest purity available. *Ortho*-phosphoric acid ( $\text{H}_3\text{PO}_4$ ), boric acid ( $\text{H}_3\text{BO}_3$ ), hydrochloric acid (HCl), sodium hydroxide (NaOH), monobromobimane ( $\text{C}_{10}\text{H}_{11}\text{N}_2\text{O}_2\text{Br}$ ), sodium borohydride ( $\text{NaBH}_4$ ), diethylenetriaminepentaacetic acid anhydride ( $\text{C}_{14}\text{H}_{19}\text{N}_3\text{O}_8$ ), ethylenediaminetetraacetic acid (EDTA), lugol, and Tris (hydroxymethyl) amino methane (TRIS) were purchased from Sigma (St. Louis, MO, USA). High-performance liquid chromatography-grade methanol was purchased from Scharlau Chemie (Barcelona, Spain). Filters were obtained from Millipore (Millipore Ibérica, Madrid, Spain).

The different reagents and running buffers were prepared with Milli-Q® water obtained from a Milli Q Plus system (Millipore Iberica).

### ***Tetraselmis suecica* culture conditions and cadmium treatment**

Cells of the marine microalga *T. suecica* (Kyllin) Butch were grown in natural, enriched seawater medium with the addition of inorganic nutrients without EDTA and TRIS [19]. The seawater was passed through a 0.45- $\mu\text{m}$  Millipore filter and a charcoal

column to eliminate organic chelating substances and, afterward, was sterilized at 121°C for 20 min. The salinity of the seawater was 35‰, and the initial pH of the culture was 7.8. Cultures were maintained at  $18 \pm 1^\circ\text{C}$  under a light intensity of 68  $\mu\text{E}/\text{m}^2/\text{s}$  using cool fluorescent light with a 12:12-h light:dark cycle. Natural sterile air was constantly bubbled at a flow rate of 10 L/min.

*Tetraselmis suecica* was cultured for 8 d with cadmium concentrations of 3, 7.9, 15, and 30 mg/L. Initial cell density in the assays was  $25 \times 10^4$  cells/ml. Control cultures without cadmium were included. Each experiment was carried out in triplicate.

The cadmium concentration corresponding to the median effective concentration (EC50) for this microalga was estimated previously [20] as  $7.9 \pm 1$  mg/L of cadmium after 6 d of exposure to this metal. Therefore, the cadmium concentration of 7.9 mg/L was selected for the assay, and two concentrations above (15 and 30 mg/L) and one concentration below (3 mg/L) also were assayed.

Growth of microalgal cultures was measured daily by counting culture aliquots in a Neubauer hemocytometer chamber after fixation with lugol using a light microscope. Aliquots of *T. suecica* cells were harvested by centrifugation (1,500 g for 10 min at 15°C) each day and stored at  $-20^\circ\text{C}$  until the analysis.

### **Characterization and quantification of cadmium-induced thiols**

Thiol peptides were extracted from frozen samples and derivatized with monobromobimane that specifically labels sulfhydryl-containing compounds. Separation and analysis of labeled-thiol peptides were carried out using capillary electrophoresis [18]. The equipment used was a HP3DCE (Capillary Electrophoresis System; Agilent Technologies, Waldbronn, Germany). Standards of phytochelatin, cysteine,  $\gamma$ -Glu-Cys, and GSH, derivatized in the same way as the biological samples, were used to identify the different peaks. Glutathione was used as a suitable calibrating substance for quantifying the different peaks.

### **Determination of intracellular cadmium**

A 25-ml aliquot from each microalgal culture was centrifuged at 1,500 g for 5 min, and the pellet was resuspended for 20 min in 25 ml of a solution containing 0.02 M EDTA dissolved in seawater. Afterward, the cells were centrifuged (1,500 g for 10 min

at 15°C) and washed twice with seawater. The cellular pellet was digested for 24 h with 1 ml of 15 M HNO<sub>3</sub> and 0.5 ml HClO<sub>4</sub>. The cadmium in the samples was measured by inductively coupled plasma–mass spectrometry (ICP-MS) using a VG Elemental Plasma Quad 2 ICP-MS System (VG Elemental, Offenbach, Germany) [20].

### **Stability of phytochelatins and their precursors after transferring to metal-free medium**

*Tetraselmis suecica* cells exposed to a cadmium concentration of 7.9 mg/L for 7 d were transferred to metal-free medium to monitor the persistence of phytochelatins and their precursors in cells. Cultures were carried out as described above.

Cells were harvested by centrifugation (1,500 g for 10 min at 15°C) and resuspended in a solution containing 0.02 M EDTA dissolved in seawater. Cells were kept in this solution for 10 min; afterward, cells were centrifuged (1,500 g for 10 min at 15°C) and washed twice with seawater. Washing with EDTA removed the cadmium adsorbed onto the cell surface. Cells were resuspended in metal-free seawater enriched with inorganic nutrients without EDTA and TRIS. Cultures were carried out as described above. Thiols and the intracellular concentration of the metal were monitored during the assay (as indicated above) every 2 h during the phase of light for a short time course evaluation (10 h).

### **Statistical analysis**

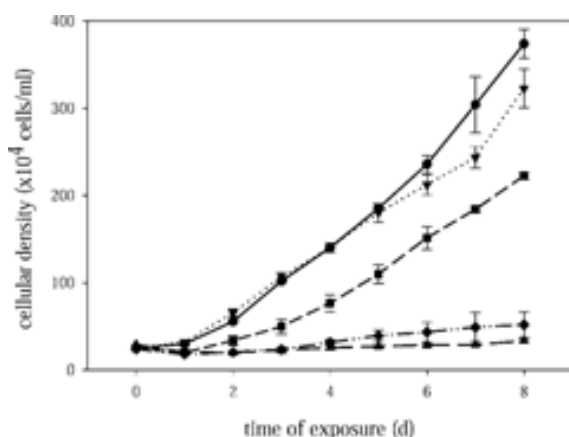
Data were analyzed using the statistical program SPSS 11.5 (SPSS Iberica, Madrid, Spain). The results were tested by oneway analysis of variance (ANOVA), and the mean values of the various treatments were compared by Duncan's multiple-range test to a level of 5%.

## **RESULTS**

### **Effect of cadmium on *T. suecica* growth**

Cultures of *T. suecica* exposed to different cadmium concentrations (3, 7.9, 15, and 30 mg/L) showed significant differences in their growth. Growth inhibition was observed in all cultures exposed to cadmium with respect to the control culture without

metal. The ANOVA test ( $p > 0.05$ ) showed that this inhibitory effect on growth was significant in all cultures. The inhibition was proportional to cadmium concentration: As cadmium concentration increased in the medium, growth decreased (Fig. 1). A stimulating effect (hormesis) was observed during the first 4 d in cultures exposed to the lower cadmium concentration (3 mg/L), but later, the growth decreased significantly. Growth was not observed in cultures containing the highest cadmium concentration (30 mg/L).



**Fig. 1.** Growth of *Tetraselmis suecica* cells with the cadmium concentrations assayed. —●— = control; —▲— = 3 mg/L; —■— = 7.9 mg/L; —◆— = 15 mg/L; —▲— = 30 mg/L.

### Characterization and quantification of thiols in *T. suecica* cells by a capillary zone electrophoresis technique

A capillary zone electrophoresis technique was used to measure GSH,  $\gamma$ -Glu-Cys, the amino acid cysteine, and phytochelatins previously derivatized with monobromobimane [18]. Desglycyl-peptides also were detected. Using this technique, all the thiols mentioned previously could be analyzed in the same run.

#### GSH, $\gamma$ -Glu-Cys, and cysteine

The synthesis of these low-molecular-weight thiols depends on the concentration of cadmium added to the medium (Fig. 2).

*Tetraselmis suecica* cells treated with the lowest cadmium concentration (3 mg/L) showed decreased GSH content during the first day of exposure. Later, GSH increased until it reached the initial content and then remained steady without significant differences ( $p < 0.05$ ) until day 6, increasing slightly at the end of the culture period. However, GSH content never exceeded the amount synthesized by the control or the amount presented on day 0, with  $202.48 \pm 41.87$  amol -SH/cell (where a =  $10^{-18}$  mol

and -SH refers to thiol group) being the highest GSH content (the last day of culture) detected in cultures with 3 mg/L of cadmium. The  $\gamma$ -Glu-Cys did not show significant differences along the assay in cells exposed to this low cadmium concentration (3 mg/L), with  $121.14 \pm 61.72$  amol -SH/cell being the highest amount measured, detected on the fourth day of culture. Nevertheless, cysteine showed an increase higher than that of GSH and  $\gamma$ -Glu-Cys, reaching a value of  $316.38 \pm 17.395$  amol -SH/cell on the seventh day of culture.

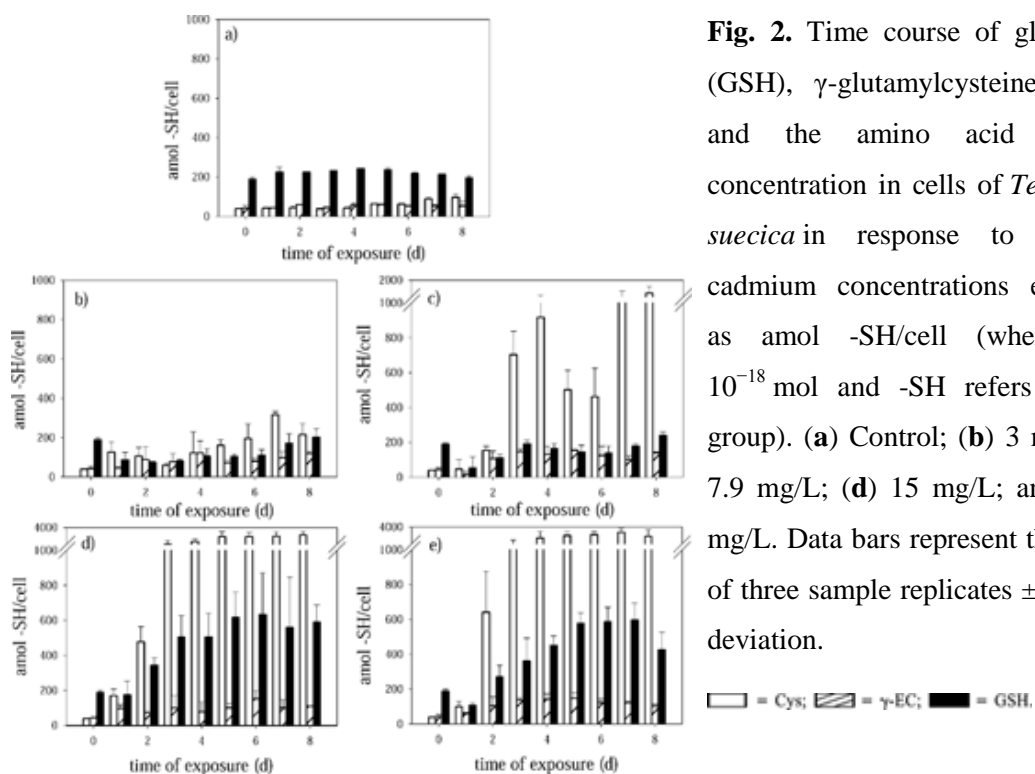
Cultures containing 7.9 mg/L of cadmium showed decreased amounts of GSH and  $\gamma$ -Glu-Cys on the first day; after 2 d, the concentration of these peptides remained without significant changes ( $p < 0.05$ ) until the last day of culture, when the amount of GSH increased slightly and reached  $240.98 \pm 18.56$  amol -SH/cell. The concentration of GSH did not exhibit significant differences among control cultures and those exposed to 3 and 7.9 mg/L of cadmium (Fig. 2a–c).

The highest GSH levels were obtained in cultures treated with 15 mg/L of cadmium, exceeding the levels observed on day 0 from the second day of treatment and reaching the highest amounts of GSH of the four cadmium concentrations assayed. The highest concentration of this small peptide was reached on the sixth day of culture ( $633.06 \pm 238.82$  amol -SH/cell), being 3.3-fold higher than the levels observed on day 0 and 2.9-fold higher with respect to the control on the same day of culture (Fig. 2d).

The concentration of GSH in cultures exposed to 30 mg/L of cadmium decreased on the first day, but during later days, the levels of this peptide increased substantially with respect to cultures treated with the lower cadmium concentrations, decreasing only on the last day (Fig. 2e). The highest amount was reached after 7 d of culture, being 3.1-fold higher than that on day 0 and 2.8-fold higher with respect to the control. Therefore, the levels of GSH in cells treated with 15 and 30 mg/L of cadmium exceeded substantially the amount measured in control cultures.

The amino acid cysteine, however, exceeded in all cultures the concentrations reached in controls. The amount reached by this amino acid was higher at the higher cadmium concentrations (Fig. 2).





**Fig. 2.** Time course of glutathione (GSH),  $\gamma$ -glutamylcysteine ( $\gamma$ -EC), and the amino acid cysteine concentration in cells of *Tetraselmis suecica* in response to different cadmium concentrations expressed as amol -SH/cell (where a =  $10^{-18}$  mol and -SH refers to thiol group). (a) Control; (b) 3 mg/L; (c) 7.9 mg/L; (d) 15 mg/L; and (e) 30 mg/L. Data bars represent the means of three sample replicates  $\pm$  standard deviation.

The concentrations of the amino acid cysteine increased from the first day of culture in all the concentrations assayed. This amino acid increased substantially in cells exposed to 15 and 30 mg/L of cadmium and exceeded by 74.7- and 86.1-fold the amount recorded on day 0, reaching a concentration of  $2,915.02 \pm 487.12$  and  $3,359.54 \pm 499.15$  amol -SH/cell, respectively (Fig. 2d and e). The amino acid cysteine also increased significantly in cultures treated with 7.9 mg/L of cadmium after the second day, reaching an amount on the last day that was 35.1-fold higher than that on day 0. Cysteine began to increase on the fourth day of culture in cells treated with 3 mg/L of cadmium. Cysteine reached a concentration that was 30.1- and 38.2-fold higher than that of the control in cultures containing 15 and 30 mg/L of cadmium, respectively, on the days (8 and 7, respectively) in which this amino acid reached the highest values. This amino acid exhibited significant differences compared with  $\gamma$ -Glu-Cys and GSH in cultures with 7.9, 15, and 30 mg/L of cadmium ( $p > 0.05$ ).

The dipeptide  $\gamma$ -Glu-Cys presented the fewest changes at the cadmium concentrations tested. After the second day, in the cultures containing 3 and 7.9 mg/L of cadmium, this peptide remained without significant differences ( $p < 0.05$ ), with the highest concentration of  $\gamma$ -Glu-Cys measured being in cultures treated with 7.9 mg/L of cadmium when a concentration of  $152.63 \pm 45.01$  amol -SH/cell was reached. In the

same way, in cultures exposed to 15 and 30 mg/L of cadmium, this small peptide remained without major variations.

### **Phytochelatins**

The time course analysis showed that the concentrations of phytochelatins and their number of subunits depend on the amount of cadmium added to the culture and on the time of exposure (Fig. 3). Synthesis of phytochelatins was not detected with the technique applied in the control and at day 0 of each culture.

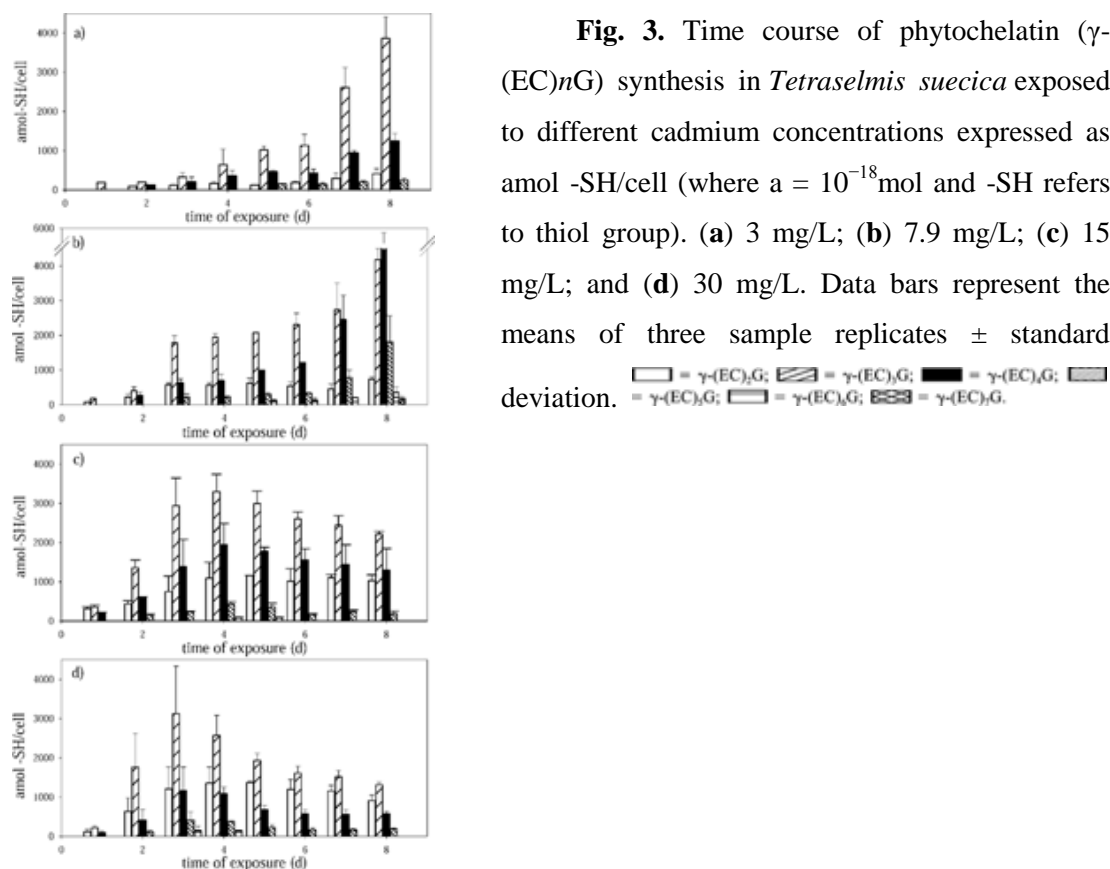
Synthesis of phytochelatins showed a similar pattern in cultures with 3 and 7.9 mg/L of cadmium (Fig. 3a and b). The levels of the different types of phytochelatins in cultures treated with 3 and 7.9 mg/L of cadmium increased progressively in the course of time. The  $(\gamma\text{-EC})_2\text{G}$  to  $(\gamma\text{-EC})_5\text{G}$  were detected under both assayed cadmium concentrations.

The rate of  $(\gamma\text{-EC})_3\text{G}$  accumulation was higher than that of any other type of phytochelatins in cells exposed to the lowest cadmium concentration studied (3 mg/L) for all days of culture, reaching a value of  $3,859.60 \pm 553.11$  amol -SH/cell. However, in cultures treated with 7.9 mg/L of cadmium,  $(\gamma\text{-EC})_4\text{G}$  increased substantially after 7 d of culture, exceeding the amount of  $(\gamma\text{-EC})_3\text{G}$  and reaching a concentration of  $4,540.19 \pm 1,042.13$  amol -SH/cell. The peptide  $(\gamma\text{-EC})_4\text{G}$  exceeded by 17-fold the initial amount of this type of phytochelatin. A  $(\gamma\text{-EC})_6\text{G}$  was detected from the fifth day of culture in cells exposed to 7.9 mg/L of cadmium, and a  $(\gamma\text{-EC})_7\text{G}$  appeared on the last day of culture in this same cadmium concentration.

Phytochelatins of cultures exposed to 15 mg/L of cadmium increased until the fourth day of culture, with the amount of  $(\gamma\text{-EC})_3\text{G}$  being significantly higher than that of the other types of phytochelatins ( $p > 0.05$ ), reaching a concentration of  $3,299.87 \pm 438.17$  amol -SH/cell (Fig.3c). After a 4-d culture, phytochelatin levels decreased gradually, coincident with a considerable increase in GSH and cysteine (Fig. 2d).

As in cultures exposed to 15 mg/L of cadmium, cells of *T. suecica* treated with the highest cadmium concentration (30 mg/L) showed a decrease in the concentration of phytochelatins after 3 d of culture (Fig. 3d). At the same time, an increase in the concentration of cysteine and GSH was measured. The amount of  $(\gamma\text{-EC})_3\text{G}$ , as in cultures exposed to 3 and 15 mg/L of cadmium, was significantly higher than that of the

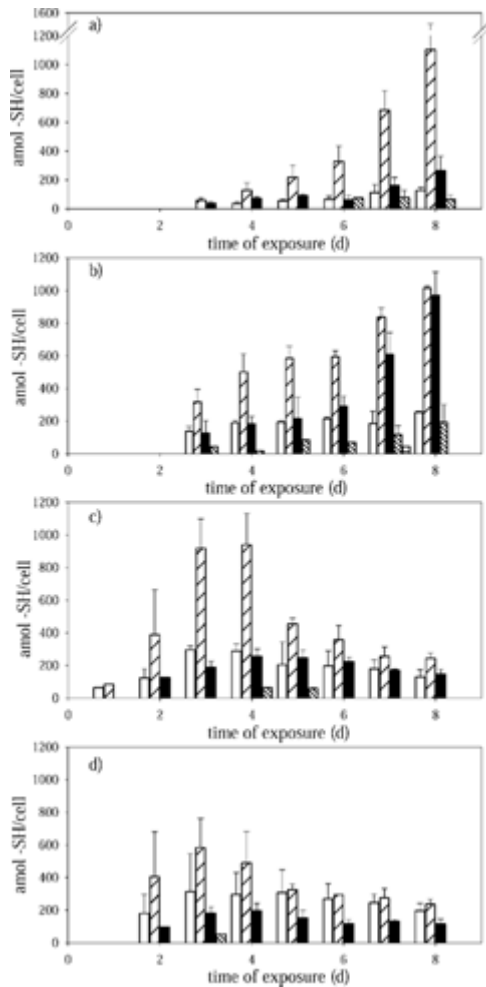
other types of phytochelatins ( $p > 0.05$ ), reaching a concentration of  $3,123.71 \pm 1,210.354$  amol -SH/cell.



### Desglycyl-phytochelatins

The capillary zone electrophoresis technique used for the quantification and characterization of the different thiol compounds allowed the identification and separation of desglycyl-phytochelatins [18].

These types of peptides were not detected during the two first days of culture in the cells exposed to 3 and 7.9 mg/L of cadmium, although in cultures exposed to 30 mg/L of cadmium, these peptides were already detected on the second day (and from the first day in the cultures treated with 15 mg/L of cadmium) (Fig. 4).

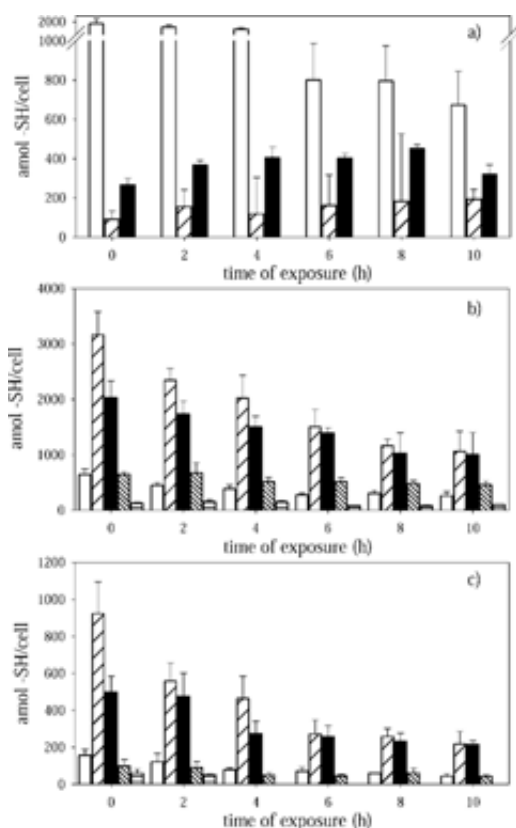


**Fig. 4.** Time evolution of desglycyl-phytochelatin ( $\gamma$ -(EC) $n$ ) in cultures of *Tetraselmis suecica* exposed to different cadmium concentrations expressed as amol -SH/cell (where  $a = 10^{-18}$  mol and -SH refers to thiol group). (a) 3 mg/L; (b) 7.9 mg/L; (c) 15 mg/L; and (d) 30 mg/L. Data bars represent the means of three sample replicates  $\pm$  standard deviation.

□ =  $\gamma$ -(EC) $_2$ ; ▨ =  $\gamma$ -(EC) $_3$ ; ■ =  $\gamma$ -(EC) $_4$ ;  
 ▩ =  $\gamma$ -(EC) $_5$ ; ▭ =  $\gamma$ -(EC) $_6$

The time course of these desglycyl-peptides followed the same pattern as that of phytochelatin in the different cadmium concentrations. The concentration of desglycyl-phytochelatin in cultures treated with 3 and 7.9 mg/L of cadmium increased gradually during the assay. However, in the cultures exposed to 15 and 30 mg/L of cadmium, the different types of desglycyl-peptides decreased from the fourth and third day, respectively.

The concentration of ( $\gamma$ -EC) $_3$  significantly exceeded ( $p > 0.05$ ) that of the other types of desglycyl in all the concentrations of cadmium assayed, with  $1,102.048 \pm 369.166$ ,  $1,014.327 \pm 13.80$ ,  $939.445 \pm 192.268$ , and  $582.931 \pm 179.351$  amol -SH/cell being the highest levels of ( $\gamma$ -EC) $_3$  reached in the cultures treated with 3, 7.9, 15, and 30 mg/L of cadmium, respectively.



**Fig. 5.** Time evolution of thiol pools on resuspension of cells in medium without metal (cells were previously exposed to 7.9 mg/L of cadmium for 7 d) expressed as amol -SH/cell (where a =  $10^{-18}$  mol and -SH refers to thiol group. (a) Time course of the amino acid cysteine and precursors of phytochelatin, glutathione (GSH), and  $\gamma$ -glutamylcysteine ( $\gamma$ -EC).

( $\gamma$ -EC),  $\square$  = Cys;  $\text{▨}$  =  $\gamma$ -EC;  $\blacksquare$  = GSH. (b) Time course of phytochelatin ( $\gamma$ -(EC)<sub>n</sub>G),  $\square$  =  $\gamma$ -(EC)<sub>1</sub>G;  $\text{▨}$  =  $\gamma$ -(EC)<sub>2</sub>G;  $\text{▩}$  =  $\gamma$ -(EC)<sub>3</sub>G;  $\blacksquare$  =  $\gamma$ -(EC)<sub>4</sub>G;  $\text{▨}$  =  $\gamma$ -(EC)<sub>5</sub>G;  $\text{▩}$  =  $\gamma$ -(EC)<sub>6</sub>G. (c) Time course of desglycyl-phytochelatin ( $\gamma$ -(EC)<sub>n</sub>),  $\square$  =  $\gamma$ -(EC)<sub>2</sub>;  $\text{▨}$  =  $\gamma$ -(EC)<sub>3</sub>;  $\blacksquare$  =  $\gamma$ -(EC)<sub>4</sub>;  $\text{▨}$  =  $\gamma$ -(EC)<sub>5</sub>. Data bars represent the means of three sample replicates  $\pm$  standard deviation.

### Stability of phytochelatin after transferring to a metal-free medium

Cells of *T. suecica* exposed to 7.9 mg/L of cadmium for 7 d were transferred to metal-free medium. Phytochelatin, desglycyl-phytochelatin, GSH,  $\gamma$ -Glu-Cys, and cysteine were monitored for 10 h. Phytochelatin, desglycyl-phytochelatin, and the amino acid cysteine decreased gradually with time (Fig. 5). This reduction mainly affected ( $\gamma$ -EC)<sub>3</sub>G and ( $\gamma$ -EC)<sub>4</sub>G, with these being the most abundant type of phytochelatin at the beginning of the assay (Fig. 5b). The decrease in desglycyl-peptides also mainly affected ( $\gamma$ -EC)<sub>3</sub> and ( $\gamma$ -EC)<sub>4</sub>. A decrease in the concentration of phytochelatin occurred simultaneously with a cellular increase in GSH and  $\gamma$ -Glu-Cys (Fig. 5a). These small peptides exceeded by 1.6- and 2.1-fold, respectively, the concentration at the beginning of the assay, although the concentration reached by  $\gamma$ -Glu-Cys was 2.6-fold lower than that of GSH.

The analysis of intracellular cadmium showed that the intracellular concentration of this metal was constant during the assay (10 h).

## DISCUSSION

Cadmium toxicity was clearly demonstrated; however, what generally is accepted to be deleterious to living organisms provoked a hormesis effect in cultures of the marine microalga *T. suecica* exposed to 3 mg/L. Also, Lane and Morel [21] provided evidence of a biological role for cadmium in the marine diatom *Thalassiosira weissflogii*. The addition of cadmium to zinc-limited cultures increased the growth rate. Those authors found that this microalga expresses a cadmium-specific carbonic anhydrase that could substitute the zinc enzyme TWCA1 (*T. weissflogii* carbonic anhydrase), the major intracellular carbonic anhydrase.

Marine microalgae are among the organisms that tolerate higher concentrations of this metal. *Tetraselmis suecica* is a good example of a tolerant microalga. The EC50 value obtained for the growth of this species exposed to cadmium was 7.9 mg/L cadmium [20]. This value indicates that this microalgal species is one of the most tolerant to this metal. One of the reasons for this tolerance is the ability of this species to synthesize phytochelatins and related molecules. Different reports have shown that biosynthesis of phytochelatins seems to be one of the main tolerance mechanisms to metals, with cadmium being one of the main inducers [22,23]. In fact, a previous analysis showed that approximately 87% of the bioaccumulated cadmium was bound by phytochelatins when this microalga was exposed to 6 mg/L of cadmium for 8 d [24]. The concentration of thiol groups in *T. suecica* also increased as cadmium increased in the medium, with this increase being higher with the higher cadmium concentrations assayed [20]. These results agree with the increase in phytochelatins in the cultures treated with 3 and 7.9 mg/L of cadmium. The synthesis of phytochelatins allows a great capacity to bind cadmium that prevents the metal from affecting the cellular targets. Ahner et al. [25] exposed eight species of eukaryotic microalgae to increased concentrations of cadmium. Those algae exhibited a similar response, and those authors observed that the concentration of phytochelatins increased with that of cadmium. This fact is common to the species of phytoplankton investigated; therefore, it could be considered to be a general response.

The present results, however, showed that phytochelatin synthesis is an effective defense mechanism in the response to cadmium, but only at metal concentrations less than the EC50. Cultures of *T. suecica* exposed to the higher cadmium concentrations

(15 and 30 mg/L) showed a decrease in the levels of phytochelatins after 4 and 3 d of exposure, respectively; nevertheless, the number of thiol groups did not decrease [20]. Although growth was totally inhibited in cultures exposed to the higher cadmium concentrations, synthesis of the amino acid cysteine was high (and synthesis of GSH was less), reaching a concentration 86.1-fold higher than that on day 0 on the seventh day of culture. In the same way, cells treated with 15 mg/L of cadmium showed a substantial increase in cysteine (74.7-fold higher than on day 0), although growth was near zero. At these high cadmium concentrations, this amino acid might be acting as main chelator for cadmium. Previously, intracellular cadmium in cells of *T. suecica* exposed to 30 mg/L was shown to exceed considerably the amount of intracellular cadmium existing in the cultures with lower concentrations from the first day [20]; therefore, cadmium was bioaccumulated in this culture. Bearing this in mind, the present study demonstrated that cysteine might be the principal chelator of cadmium when the concentration of this metal is so high that synthesis of phytochelatins is restricted. The role of cysteine in metal detoxification was studied in several microalgae and plants. Exposure to cobalt ions resulted in increased citrate and cysteine levels in cells of *Crotalaria cobalticola*, where the synthesis of phytochelatins was not induced [12]. The synthesis of cysteine in a tolerant strain of *Scenedesmus acutus* increased with the cadmium concentration assayed, reaching an amount that was fivefold higher than that of the wild strain in the culture with the higher cadmium concentration (27  $\mu$ M); a decrease in phytochelatins also was observed both in the tolerant and the wild strains when cadmium concentrations were increased [26]. Ahner et al. [14] reported that cysteine increased with cadmium concentration and exceeded the concentration of GSH at the highest cadmium concentrations in the different species of microalgae assayed. Transgenic plants of *Arabidopsis* with altered GSH levels were unable to synthesize phytochelatins; however, in these plants, the steady state of cysteine concentration increased twofold in response to cadmium exposure [27]. Consequently, as the concentration of cadmium overcomes a specific value (possibly the EC50 for growth of the species), the toxic effect of cadmium blocks the synthesis of phytochelatins, with cysteine being the main chelating agent. However, cysteine presents a lower capability of chelating the metal; therefore, cysteine would not avoid cadmium toxicity when a high intracellular concentration of this metal exists. Therefore, in cultures exposed to 15 and 30 mg/L of cadmium, growth inhibition is not prevented.

The data indicate that the halt in the synthesis of phytochelatin in cultures containing the higher cadmium concentration result from an inhibition in phytochelatin synthase, because the synthesis of cysteine and GSH continued and the concentration of these two molecules increased. One explanation for this result is deduced from the catalytic characteristics of phytochelatin synthase [28]. This enzyme requires free GSH for the catalysis. In cultures with higher concentrations of cadmium, the union of this metal blocks all available GSH. This indicates an increase in GSH synthesis (Fig. 2d and e) to compensate for the one that is blocked, but this synthesis is insufficient to generate free GSH. Therefore, no synthesis of phytochelatin occurs. These processes provoke that the cadmium remains free and causes the toxicity.

It is necessary to emphasize that in contrast to what happens with the synthesis of phytochelatin, the synthesis of cysteine is very high in cultures with a high cadmium concentration, indicating that the enzymes involved in the synthesis of this molecule are very active. For example, in plants of *Arabidopsis thaliana* exposed to cadmium, expression of the cytosolic *O*-acetyl-serine(thiol)lyase gene is high [13]. Therefore, in the cells of *T. suecica*, despite being exposed to high cadmium concentrations, the metabolism of sulfur (assimilation pathway) continues to be high. Under these conditions, cysteine becomes the mayor thiol in the cells.

Glutathione levels in cultures exposed to cadmium concentrations less than the EC50, however, recuperated as the culture progressed, reaching values similar to (or only slightly higher than) those of the control. From this, it is deduced that the capacity of GSH synthesis is sufficient to compensate for its loss because of the synthesis of phytochelatin and the one that has been blocked by cadmium complexation. This maintains the GSH levels that are needed for other functions, allowing these cultures to continue growing, although with a lower growth rate than that of the control (Fig. 1).

*Tetraselmis suecica* also showed decreased GSH on the first day of culture with respect to that on day 0 in all cultures exposed to cadmium. This same pattern was observed in most algae and in plants [23]. In fact, in plant cells, a general tendency is a concomitant disappearance of GSH and the appearance of phytochelatin [29]. This general response is explained by the demands of GSH for synthesis of phytochelatin. Also, cadmium provokes the production of reactive oxygen species that induce the



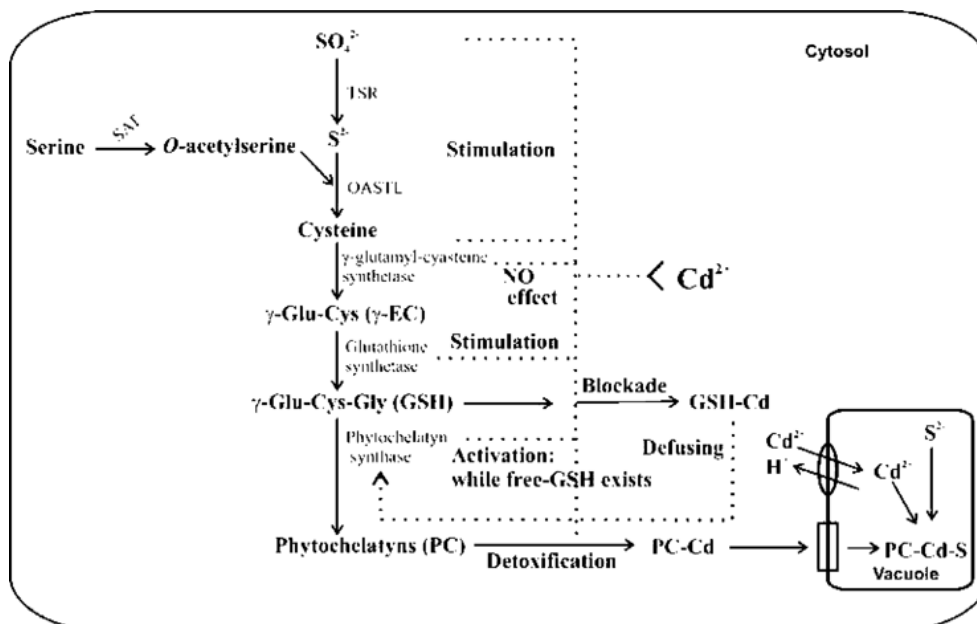
activity of antioxidant enzymes, such as superoxide dismutase, or low-molecular-weight antioxidants, such as GSH [30].

It is interesting that the level of  $\gamma$ -Glu-Cys remained without significant increases in all cultures, even those with the highest cadmium concentration, in which both GSH and cysteine presented a strong increase. This seems to indicate that the activity of  $\gamma$ -Glu-Cys synthetase did not increase greatly because of cadmium in *T. suecica* cells; therefore,  $\gamma$ -Glu-Cys levels remained constant in all cultures. This same effect was observed in plants of *Brassica juncea*, in which the transcript amount for  $\gamma$ -Glu-Cys synthetase did not change significantly after cadmium exposure [31]. Although cadmium did not induce higher expression of the gene for this enzyme, its activity possibly was increased by the action of the metal; nevertheless, our results seem to indicate that this did not occur. These results confirm, as generally accepted, that  $\gamma$ -Glu-Cys synthetase is the rate-limiting step in GSH biosynthesis [32,33] and, therefore, in the synthesis of phytochelatins, because as observed in the obtained data, cysteine synthesis does not rate-limit GSH production under the conditions of toxicity for cadmium. Elevated  $\gamma$ -Glu-Cys synthetase activity (i.e., overexpression) was shown to correlate with cadmium resistance in cultured tomato cells [34] and in *B. juncea* [35].

According to the present results, a model for the interaction of cadmium with the enzymes involved in the biosynthetic pathway of phytochelatins in the microalga *T. suecica* is presented in Figure 6.

Phytochelatins and cysteine are important cellular components involved in mechanisms of tolerance to cadmium, with the intracellular level of these molecules being regulated by the concentration of this metal in the medium. The size of the phytochelatins, however, also is an important aspect for the reduction of cadmium toxicity. The ability of an organism to synthesize phytochelatins with a greater number of subunits would allow it to tolerate a higher concentration of cadmium; therefore, it would increase the EC50. In the case of *T. suecica*, phytochelatins with up to seven subunits were detected. The most abundant phytochelatin had four subunits (Fig. 3b). These phytochelatins are produced in cultures with the cadmium concentration that implies the EC50 for the growth of this species. The elevation of this metal concentration exceeds the homeostatic characteristics of *T. suecica* (reduction in the synthesis of phytochelatins is produced), and growth is inhibited.

A second family of thiol peptides was characterized in *T. suecica*, the desglycyl-phytochelatin with the general structure  $(\gamma\text{-Glu-Cys})_n$ . The desglycyl-phytochelatin were described previously in yeast [36] and in plants [9,37]. Meuwly et al. [37] described three families of thiol peptides in maize treated with cadmium:  $(\gamma\text{-Glu-Cys})_n$ ,  $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ , and  $(\gamma\text{-Glu-Cys})_n\text{-Glu}$  [37]. Also, *Rubia tinctorum* exposed to different metals synthesized  $(\gamma\text{-Glu-Cys})_n\text{-Gly}$  and  $(\gamma\text{-Glu-Cys})_n$  [38]. Desglycyl-phytochelatin appeared on exposure to metals; therefore, as phytochelatin, they could act in the detoxification of metals. However, desglycyl-peptides and phytochelatin showed a similar pattern of synthesis in *T. suecica* (Figs. 3 and 4), with the desglycyl peptides concentration being substantially lower than that of phytochelatin. Today, doubts exist about the biosynthetic origin of these molecules. One possible origin might be a process of degradation of phytochelatin because of the action of a carboxypeptidase [7]. Nevertheless, the data obtained in the present work indicate a pattern of synthesis correlated with that of the phytochelatin, which might indicate that the desglycyl-phytochelatin would be a residual activity of phytochelatin synthase, which also might polymerize subunits of  $\gamma\text{-Glu-Cys}$ . When the activity of this enzyme decreased in cultures exposed to 15 and 30 mg/L of cadmium, decreasing the amount of phytochelatin, the amount of desglycyl-phytochelatin also decreased in the same way.



**Figure Fig. 6.** Schematic representation of cadmium interaction with the different enzymes involved in the biosynthetic pathway of phytochelatin. For a detailed explication, see

the text.  $\gamma$ -EC =  $\gamma$ -glutamylcysteine; GSH =  $\gamma$ -glutamylcysteine-Gly; OASTL = *O*-acetylserine thiol lyase; PC = phytochelatin; SAT = serine acetyl transferase; TSR = thiosulfate reductase.

In cultures that were placed in a medium without cadmium, rapid loss of phytochelatin from the cells occurs after exposure to an EC50 concentration of this metal. After 10 h, the content of phytochelatin diminished to 50% of the initial concentration (Fig. 5). The quantity of cysteine also diminished, but the quantities of GSH and  $\gamma$ -Glu-Cys increased. Loss of phytochelatin also occurred in *T. weissflogii* and *Phaeodactylum tricornutum* previously exposed to cadmium and lead, respectively [39,40]. The decrease in levels of phytochelatin indicates an excretion or degradation process; in fact, in both plants and yeast, phytochelatin–cadmium complexes are sequestered into vacuoles. However, no excretion of metals was observed in *T. suecica* during the assay (10 h). These data seem to indicate a degradation process of phytochelatin, with the metal remaining inside the vacuoles. Once the metal is excreted to the vacuoles, the phytochelatin would be degraded, decreasing their levels, because the activity of phytochelatin synthase also would be lower.

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