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Biosynthesis of phytochelatins and arsenic accumulation in the marine microalga *Phaeodactylum tricornutum* in response to arsenate exposure

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Abstract

The arsenate-induced synthesis of phytochelatins (PC), intracellular cysteine-rich metal-binding peptides, and its relationship with toxicity and with As accumulation in the cell have been studied in laboratory cultures of the marine microalga *Phaeodactylum tricornutum*. The time course of cellular PC and As in short-term exposures showed that the involvement of PC in the As detoxification as well as the pathway of cellular As depend on the extent of As accumulation and on the rate of PC synthesis. At arsenate concentrations causing As accumulation at a rate exceeding that of PC synthesis, cells seem to activate a mechanism of release of As mainly in a chemical form not complexed with PC. At arsenate concentrations at which the synthesis of PC occurs at a rate sufficient to allow a significant portion of As accumulated in the cell to be bound, the fate of cellular As seems to be mainly controlled by PC. The occurrence of these different pathways of As detoxification was discussed to explain the pattern of cellular As and PC in cells grown for three days at growth-inhibitory and at no growth-inhibitory concentration of arsenate.

Introduction

Arsenic is a toxic element which is widespread in the environment. Its presence is due to both natural sources, such as volcanic action, and anthropogenic activities, such as smelting industry, burning of fossil fuels and use of arsenical pesticides in agriculture (Nriagu 1994). Arsenic can exist in several oxidation states and chemical forms and its toxicity depends on its chemical speciation. The dominant species of arsenic in aquatic systems is arsenate, which is the thermodynamically stable form of inorganic arsenic in oxygenated waters (Edmonds & Francesconi 1993). Other inorganic species, such as arsenite, and a variety of organoarsenicals, such as methylated arsenic species, have been also detected in the aquatic environments, their presence being correlated with biological activity (Anderson & Bruland 1991; Edmonds & Francesconi 1997). Knauer *et al.* (1999) report that arsenate is more toxic to phytoplankton communities than arsenite or methylated arsenic compounds, so algae can be the target of arsenic toxicity in polluted aerobic aquatic environments. Arsenate is an analogue of phosphate and is taken up by the cell through the phosphate uptake system (Ullrich-Eberius *et al.* 1989). Inside the cell, arsenate can compete with phosphate for binding by ADP, leading to the formation of an unstable ADP-As, with the loss of an important cellular energy source (Meharg 1994).

Many studies on arsenic–phytoplankton interactions have regarded the mechanisms of biotransformation of arsenate into arsenite or methylarsenicals (Takimura *et al.* 1996; Kaise *et al.* 1999; Hasegawa *et al.* 2001), but studies

regarding other constitutive mechanisms of arsenate detoxification potentially operating in these organisms are lacking. A widespread detoxification mechanism, developed by plants and algae to avoid metal poisoning, involves intracellular glutathione-related peptides. These compounds, named phytochelatins (PC) are cysteine-rich peptides with general structure (γ-Glu–Cys)*n*-Gly, with *n* values generally ranging from 2 to 6 (Grill *et al.* 1985). They are enzymatically synthesized by a specific transpeptidase, the phytochelatin synthase, which is activated by the presence of metal ions and uses glutathione as substrate (Grill *et al.* 1989). Their detoxifying function consists in the ability to bind metals in stable complexes, which effectively reduce the intracellular concentration of potentially toxic free metal ions. Although a wide literature describes the synthesis and the detoxifying function of PC in plants and algae in response to heavy metal ions, such as Cd, Cu, Hg, and Pb (Zenk 1996; Cobbett 2000), only recently the ability of arsenate anion to trigger the formation of PC has been demonstrated in a variety of terrestrial plants (Sneller *et al.* 1999; Schmöger *et al.* 2000; Hartley-Whitaker *et al.* 2002; Bleeker *et al.* 2003; Zhao *et al.* 2003; Reina *et al.* 2005) and in a freshwater green alga (Pawlik-Skowronska *et al.* 2004). To our knowledge, no studies have been reported on the arsenate-induced PC synthesis in marine phytoplanktonic algae, despite their importance as first trophic level in the food web.

The aim of the present study was the investigation of the capability of a unicellular marine alga, the diatom *Phaeodactylum tricornutum*, to activate the PC synthesis in response to arsenate. The toxic action of arsenate anion on the growth, the kinetics of cellular PC formation and the relationship between dissolved arsenate and cellular PC or As accumulation are reported and discussed.

Materials and methods

Stock cultures of the unicellular marine diatom *P. tricornutum* Bohlin (Bacillariophyceae), grown in axenic conditions, at 21 °C, using continuous illumination under fluorescent daylight (100 μmol photons m^{-2} s^{-1}), were maintained in exponential growth phase by inoculating every 7 days into a fresh culture medium. The growth medium was natural seawater enriched with the f/2 enrichment solution for microalgal cultures (Guillard 1975) at one–fifth the normal trace metal concentration and without Cu and Zn. Calculated amounts of algae were collected by filtration (1.2 μm membrane filter, Sartorius) from exponentially growing 4-days old cultures and used for arsenate exposure experiments. Living cells were counted by means of a Thoma counting chamber under a microscope. Culture medium used for arsenate exposure experiments was the chemically defined artificial seawater medium Aquil (Price *et al.* 1991) without addition of the metal stock solution, and at 0.5 μM phosphate concentration.

Arsenic-treated algae were collected by filtration onto 1.2 μm membrane filters, re-suspended in 1.5 ml of 0.1 M HCl/5 mM DTPA and disrupted by sonication (Sonopuls Ultrasonic Homogenizer, Bandelin) for 3 min with a repeating duty cycle of 0.3 s, in an ice bath. The cellular homogenate was centrifuged (35,000 *g*, 60 min) and the supernatant was used for the determination of non-protein thiols.

Non-protein thiols were separated and quantified by high performance liquid chromatography (HPLC) after derivatization with the fluorescent tag monobromobimane (mBrB) (Fahey & Newton 1987) according to the following procedure. 200 μl of the sample was added to 140 μl of 0.7 mM TCEP in 200 mM HEPPS/5 mM DTPA, pH 8.2, in order to reduce oxidized thiol groups. After 15 min of incubation, 20 μl of 10 mM mBrB in acetonitrile were added and the reaction was allowed to proceed for 15 min in the dark at 45 °C. Afterwards, 20 μl of 100 mM cysteine were added and, 15 min later, the reaction was stopped by adding 20 μl of 1 M MSA. The derivatized samples were stored in the dark at +4 °C until HPLC analysis. Blank samples were used to identify the reagent peaks. The derivatized γ-glutamyl peptides were separated on a reverse-phase column by using a linear gradient from 10 to 30% acetonitrile in 0.1% TFA for 50 min at a flow rate of 1 ml min^{-1}. Retention time of phytochelatins was checked as previously reported (Morelli & Scarano 2001) and their quantification was obtained from the relationship peak area vs. concentration of GSH standard solutions. PC were quantified as γ-Glu–Cys units. The HPLC system consisted of two Shimadzu LC-10AD pumps, a Rheodyne 7725 injection valve equipped

with a 100 μl loop, a Beckman Ultrasphere C-18 reverse-phase column (5 μm, 250 mm $\times$ 4.6 mm) and a fluorescence detector (RF-10AXL, Shimadzu) set at 380 nm excitation wavelength and 470 nm emission wavelength. Chromatographic data were processed using Chromatoplus software.

Cellular arsenic was measured in arsenate treated algae after digestion with a mixture of concentrated HNO_3 and H_2O_2 (2:1 v/v) at 45 °C for 16 h. Arsenic was measured by Electro-Thermal Atomic Absorption Spectrometry (ETAAS) using a Perkin Elmer 4100 ZL instrument, with heated graphite tubes atomizer (THGA) with integrated L'vov platforms.

All reagents were analytical grade: diethylenetriaminepentacetic acid (DTPA) and reduced glutathione (GSH) from Fluka; monobromobimane (mBrB), 4-(2-hydroxyethyl)-piperazine-1-propanesulfonic acid (HEPPS), tris (2-carboxyethyl) phosphine (TCEP) and hydrogen peroxide (30% solution) from Sigma; HCl and HNO_3 Suprapur grade and methanesulfonic acid (MSA) from Merck; arsenate (Na_2HAsO_4) from Carlo Erba; acetonitrile and trifluoroacetic acid (TFA), HPLC grade, from Baker. All reagents were prepared weekly and stored in the dark at +4 °C. Water was purified by a Milli-Q system (Millipore).

Results

Three-days exposure to arsenate

Cells of *P. tricornutum* (initial density, 3×10^4 cells ml^{-1}) were grown for three days in Aquil culture medium spiked with arsenate to obtain concentrations ranging from 0.01 to 1.0 μM. Assay of cellular As at the end of the exposure period showed that As accumulation in cells began to be detectable in cultures exposed to $[AsO_4^{3-}] > 0.1$ μM, then cellular arsenic increased with increasing concentration of the metalloid in the medium, up to 0.6 μM AsO_4^{3-} (Figure 1a, open symbols). HPLC analysis for sulfhydryl groups showed that the biosynthesis of phytochelatins occurred concomitantly with the accumulation of As in cells. PC cellular pool increased by following a dose–response relationship until to the value of 107 amol γ-Glu–Cys $cell^{-1}$ at 0.6 μM AsO_4^{3-}. At the investigated arsenate concentrations, all cultures grew exponentially during the entire period of exposure.

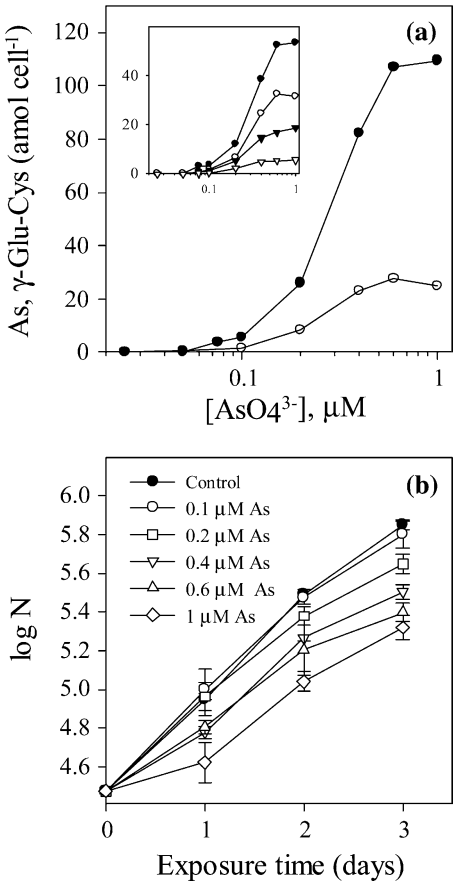

Figure 1. (a) Concentration of phytochelatins (•) and arsenic (o) in cells of *P. tricornutum* exposed for 3 days to 0.01–1.0 μM AsO_4^{3-}. Cultures were carried out in triplicate; the cells from each replicate exposure were pooled before analysis. Insert: distribution of the individual PC oligomers. PC_2 (•), PC_3 (o), PC_4 (▼), PC_5 (∇). (b) Growth curves of *P. tricornutum* in Aquil culture media containing 0.1–1.0 μM AsO_4^{3-}. Error bars correspond to the standard deviation (n = 3). N, cell number ml^{-1}.

Compared to the control (no AsO_4^{3-} added, growth rate = 1.57 ± 0.03 doublings day^{-1}) a gradual decrease of growth rate occurred at arsenate concentrations higher than 0.1 μM (Figure 1b), the concentration at which As and PC began to be detectable in the cell. The maximum inhibition of the growth rate was 35% at 1.0 μM AsO_4^{3-}.

Cellular concentration of thiol groups of PC was higher than cellular As accumulation and the mean value of the molar ratio PC-SH : As was 3.8 ± 0.5 over the whole concentration range of arsenate. *In vitro* studies on reconstructed As-PC or As-GSH complexes have shown that three thiol groups coordinate one atom of As (Pickering *et al.*

2000; Schmöger *et al.* 2000). Thus, the amount of γ-Glu–Cys units accumulated in the cell at the end of the third day of exposure could be sufficient to sequester cellular arsenic through the formation of As-PC complexes.

The cellular pool of peptides was composed by PC oligomers with *n* value from 2 to 5 (Figure 1a, insert), with a percentage of the individual peptides nearly constant in all cultures. It was calculated that the 49% of the γ-Glu–Cys peptides was polymerized as PC_2, the 30% as PC_3, the 16% as PC_4 and the 5% as PC_5, thus indicating a predominance of the pentapeptide. Accordingly, Schmöger *et al.* (2000) reported that PC_2 was the dominant oligomer in suspension cells of *Rauvolfia serpentina* in response to arsenate.

Glutathione cellular pool in exposed cells was comparable to that of As-untreated cells (mean value = 73 ± 9 amol cell^{-1}), irrespective of the different amounts of PC accumulated in the cells. Since PC are synthesized by using glutathione as substrate, this finding suggests that the cells, at the end of the 3rd day of exposure, restored the basal level of total cellular glutathione.

Kinetics of PC synthesis and As accumulation

Cells of *P. tricornutum* from 4-days old stock cultures were incubated (1×10^6 cells ml^{-1}) in fresh Aquil medium made 0.06 or 1.0 μM AsO_4^{3-}. At these concentrations, no toxic effect is expected in these short-term exposures. Aliquots of 200 ml were sampled at selected time intervals from 0 to 6 h and cells assayed for PC and As. In both experiments, arsenate induced a prompt synthesis of PC, with a maximum rate of PC formation within the first hour of exposure (Figure 2a, b, closed symbols). After this period the PC accumulation in cells exposed to 1.0 μM AsO_4^{3-} (Figure 2a) proceeded at a reduced rate. At the end of the 6th h, cellular PC was twice the amount produced in 30 min. The cellular arsenic concentration quickly reached the value of 90 amol cell^{-1}, then it continuously decreased. At the end of the experiment (6th h), the cellular As was the half of that measured after 30 min of exposure. The molar ratio PC-SH : As ranged from 0.3 to 1.2 during the exposure, so indicating that only a small amount of cellular As could be complexed by the cellular pool of PC. The pattern of As accumulation is consistent with a fast uptake of arsenate followed by a slower process of release of As, mainly in a chemical form unbound to PC. Approaching to the end of the exposure, the lowering of cellular As, together with the increase of PC, can lead to a progressively higher fraction of As potentially bound to PC.

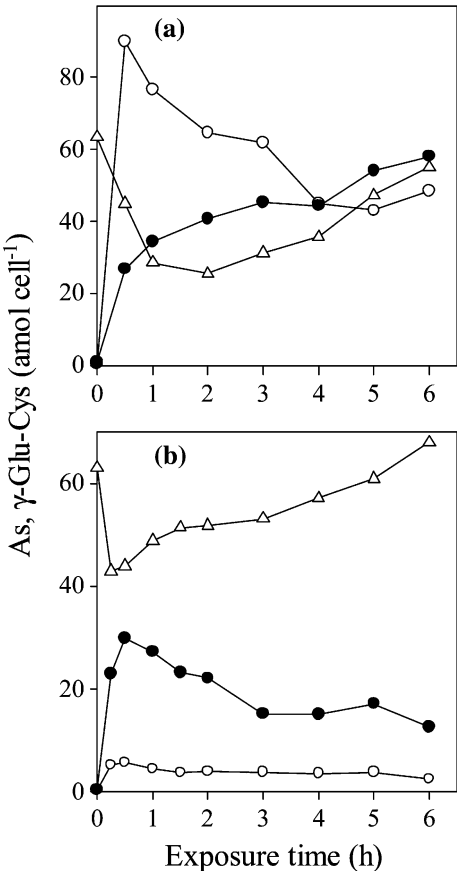

Figure 2. Time course of the concentration of phytochelatins (•), arsenic (o) and glutathione (Δ) in *P. tricornutum* cells after addition of 1.0 μM AsO_4^{3-} (a) or 0.06 μM AsO_4^{3-} (b). Cell density, 1×10^6 cells ml^{-1}.

In cells exposed to 0.06 μM AsO_4^{3-} the time course of cellular arsenic concentration showed a maximum after just 30 min of exposure, thereafter it slowly lowered. The PC cellular concentration, after reaching a maximum value during the first hour, decreased with exposure time, until to be halved at the end of the period of incubation. The molar ratio PC-SH : As, ranged from 6.3 to 3.2, indicating that the amount of thiol groups synthesized in these experimental conditions could be sufficient to complex a large amount of As entered in the cells. So, the concomitant cellular

depletion of PC and As during exposure to 0.06 μM AsO_4^{3-} indicates that arsenic accumulated in cells could be released through cellular processes involving PC. The time course of the individual peptides (Figure 3) showed that this loss involves equally all polypeptides synthesized, irrespective of their degree of polymerization. If As is bound to PC to form As-PC complexes with different stability, this suggests that the cellular loss of PC could be a process independent from the stability of As-PC complexes.

Glutathione assay (Figure 2) shows that in both kinetics the cellular concentration of glutathione decreased during the first hour of exposure, according to a rapidly induced mechanism that involves glutathione as substrate for PC synthesis. Afterwards, cellular processes to restore the glutathione cellular pool occurred. This active glutathione synthesis was also the proof that cells were in healthy state. Replicate exposure experiments, in which the time course of cellular glutathione, PC and As concentration was investigated in cultures exposed to 0.02, 0.04 and 0.80 μM AsO_4^{3-}, confirmed our results (data not reported).

In conclusion, the time course of PC and As shows that, although PC synthesis represents a cellular response of *P. tricornutum* to arsenate, the involvement of PC in the fate of cellular As seems to depend from the rate of As uptake. A more detailed investigation was performed by assaying cellular PC and As at the beginning of the exposure period (1 h) in cell suspensions exposed to several concentrations of arsenate in the range

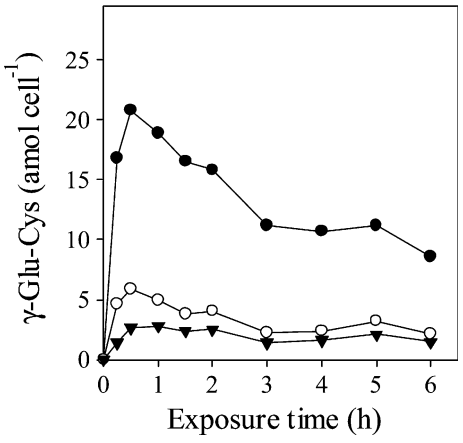

Figure 3. Time course of individual PC oligomers in *P. tricornutum* cells after addition of 0.06 μM AsO_4^{3-}. PC_2 (•), PC_3 (o), PC_4 (▼).

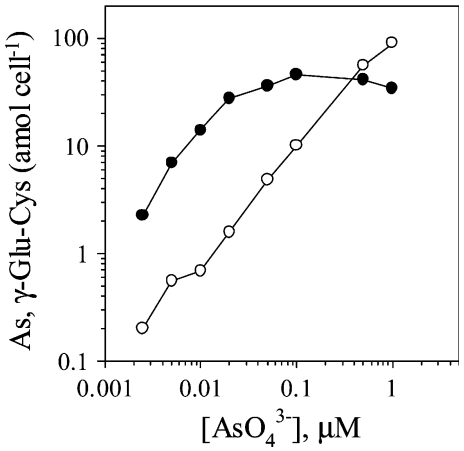

Figure 4. Relationship between arsenate concentration in the culture medium and cellular concentration of phytochelatins (•) and arsenic (o). Exposure time, 1 h. Cell density, 1×10^6 cells ml^{-1}.

0.002–1.0 μM. Figure 4 shows that cellular arsenic increases with increasing arsenate concentration in the medium, following a linear relationship during the entire range of concentrations. PC synthesis was triggered by cellular As and followed a direct dose–response relationship in the range 0.002–0.1 μM AsO_4^{3-}. In this range the thiol-PC groups are synthesized in a sufficient amount to complex As entered in the cell. At $[AsO_4^{3-}] > 0.1$ μM, PC cellular concentration levelled off becoming insufficient to sequester As entered in the cell. Under this condition of inability of the cell to synthesize more PC, cellular As seems to follow a pathway in which PC are less involved.

Discussion

Our results show that, similarly to heavy metal ions Cd, Pb and Cu (Morelli & Scarano 2001, 2004), arsenate anion induces the synthesis of PC in the marine diatom *P. tricornutum*. Although it is well documented that marine phytoplanktonic algae can synthesize these detoxifying peptides in response to a variety of metal ions (Ahner *et al.* 1995; Ahner & Morel 1995), their capability to synthesize PC in response to arsenate anion has not been reported.

The time course of PC and As in cells of *P. tricornutum* exposed to arsenate shows that both the involvement of PC in the As

detoxification and the pathway of cellular As depend on the extent of As accumulation and on the rate of PC synthesis. At arsenate concentrations causing As accumulation at a rate exceeding the rate of PC synthesis, cells seem to activate a mechanism of release of As mainly in a chemical form not complexed with PC. Thus, the observed release of As must be due to other cellular mechanisms of detoxification, PC playing a minor role. Mechanisms of bio-transformation and release of arsenic by As-treated phytoplanktonic algae have been described by several authors: Hasegawa *et al.* (2001) reported that the freshwater green alga *Closterium aciculare*, during the exponential growth, takes up arsenate and releases arsenite and methylarsenicals. Similarly, Suhendrayatna *et al.* (1999) reported that As accumulation in arsenite-treated *Chlorella vulgaris* cells was followed by an active process of excretion of arsenic metabolites. The running of similar mechanisms of biotranformation of inorganic As can be an explanation of the observed release of As by cells of *P. tricornutum* under conditions of excessive arsenate uptake.

At lower arsenate concentrations, at which the synthesis of PC occurs at a rate sufficient to bind a significant portion of As accumulated in the cell, the fate of cellular As seems to be mainly controlled by PC. In the literature there is considerable evidence that at least part of the As accumulated in plant cells can be complexed with thiol groups of PC: the formation of As-PC complexes has been demonstrated in root material of *Silene vulgaris* (Sneller *et al.* 1999) and in cell cultures of *R. serpentina* (Schmöger *et al.* 2000) by using chromatographic techniques and, in the higher plants *Holcus lanatus* and *Pteris cretica*, by using inductively coupled plasma-mass spectrometry coupled with electrospray ionization-mass spectrometry (Raab *et al.* 2004). Regarding micro algae, the involvement of the thiol groups of glutathione and phytochelatins in the intracellular complexation of As has been demonstrated in the freshwater green alga *S. bacillaris* (Pawlik-Skowronska *et al.* 2004). Our data show that cells of *P. tricornutum* can synthesize PC in amounts sufficient to complex cellular As, but further work needs to demonstrate the formation of stable As-PC complexes in this alga. However, the process of PC disappearance, concomitant with a loss of cellular As, observed during 6 h exposure to 0.06 μM arsenate, could be consistent with a process in which As-PC complexes are involved. We observed in the same alga, in which the formation of stable Cd-PC and Pb-PC complexes was demonstrated (Scarano & Morelli 2002), a similar concomitant loss of cellular metal ion and PC after the transfer of metal-treated cells in metal-free seawater (Morelli & Scarano 2001). A loss of cellular content of As and PC was also observed in roots and shoots of *S. vulgaris*, in which the formation of As-PC complexes was demonstrated, after transplant in arsenate-free media (Sneller *et al.* 1999). This suggests that the disappearance of As and PC in *P. tricornutum* may be due to a process of export or, alternatively, to a process of degradation of cellular As-PC complexes.

The toxic action of arsenate on *P. tricornutum* was demonstrated by the inhibition of the growth in contaminated medium, compared with that of control cultures, and it occurred at arsenate concentrations exceeding 0.1 μM. Cellular PC began to be detectable in cells at growth-inhibitory concentration of arsenate, and they increased with the increase of the inhibitory effect. At arsenate levels corresponding to no growth-inhibitory concentration ($[AsO_4^{3-}] < 0.1$ μM), negligible amounts of PC and As were assayed in growing cells. Because at these arsenate concentrations cells accumulated As and PC in short-exposure tests, the absence of growth inhibition can be explained by a process in which arsenic is first complexed in a non toxic form by PC and then released, according to kinetics of cellular As and PC at 0.06 μM arsenate. Cells grown at arsenate levels giving rise to a high As concentration and to an insufficient PC accumulation in short-exposure tests ($[AsO_4^{3}] > 0.1$ μM), undergo an inhibition of the growth, even if an amount of PC sufficient to bind cellular As was detected at the end of 3rd day of growth. This can be explained by a transient presence of toxic As species in the cell, followed by processes of biotransformation and of complexation by PC according to kinetics of cellular As and PC at 1.0 μM arsenate.

The role of PC in As detoxification in plants has been demonstrated by the enhanced production of these thiols in tolerant clones of the grass *H. lanatus*, with respect to the non-tolerant ones (Hartley-Whitaker *et al.* 2002) and by the arsenate hypersensitivity occurring in *Cytisus striatus* (Bleeker *et al.* 2003) and *S. vulgaris* (Schat *et al.*

2002) treated with an inhibitor of PC synthesis. Our results indicate that, although cellular mechanisms other than PC are involved in the arsenate defence ability of *P. tricornutum*, the synthesis of these peptides could represent an important line of defence of this marine micro alga against arsenate stress.

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