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Phytochelatins in the Diatom *Phaeodactylum tricornutum* Bohlin: An Evaluation of their Use as Biomarkers of Metal Exposure in Marine Waters

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Abstract The feasibility of a bioassay based on the synthesis of phytochelatins to assess metal pollution in aquatic environments was evaluated by using the marine diatom *Phaeodactylum tricornutum*. Short-term incubation experiments carried out in EDTA-buffered artificial seawater showed increasing cellular phytochelatin concentration with increasing free cadmium, lead or copper in the medium, indicating that phytochelatins behave as a biomarker of exposure to the bioavailable metal fraction. A linear dose–response relationship between metal exposure and phytochelatin synthesis was found in natural seawater samples enriched with known amounts of heavy metals. Phytochelatin induction tests carried out on polluted seawater samples showed an enhanced response compared to that obtained in unpolluted seawater. This finding was found to be consistent mainly with a copper contamination.

Keywords Biomarker · Phytochelatins · *Phaeodactylum tricornutum* · Trace metals

Heavy metal pollution in estuarine and coastal waters due to human activities represents a considerable concern for the modern world. The concentrations of total dissolved cadmium, lead, copper and zinc measured in surface seawaters are in the range 0.01–0.2 nM, 0.05–0.4 nM, 1–5 nM and 1–40 nM, respectively (Millero and Sohn 1992), but they can reach values 50–100 times higher in polluted and industrialized coastal areas. Actually, marine pollution monitoring makes use of chemical and biological parameters. As the toxicity of heavy metals is dependent on their chemical form, chemical approaches have been addressed to the characterization of metal speciation. Biomarkers, enabling molecular and physiological alterations to be evaluated, may be considered an additional and suitable tool for the assessment of water quality (Ferrat et al. 2003). Compared with the chemical measurement of the dissolved metals, biomarkers give more complete information on the potential toxicity of these contaminants on living organisms. The use of detoxification mechanisms, such as the synthesis of metallothioneins, has been proposed in a great variety of species of invertebrates as biomarkers of metal exposure (Amiard et al. 2006).

A widespread mechanism of defence developed by plants and algae against metal stress involves intracellular metal-binding peptides. These compounds, named phytochelatins (PC), are cysteine-rich peptides with a general structure (γ-Glu-Cys)n-Gly, with n values generally ranging from 2 to 6 (Grill et al. 1985). PC are rapidly synthesized by a specific enzyme, phytochelatin synthase, which is activated by the presence of metal ions and uses glutathione or PC as a substrate (Grill et al. 1989). The presence of intracellular PC is an early and specific signal of metal stress, so these compounds can be considered suitable biochemical indicators for metal exposure. This is supported by the finding that enhanced cellular PC have been measured in natural populations of marine and freshwater phytoplankton collected in polluted areas (Ahner et al. 1994, 1997; Knauer et al. 1998). In the present work, we investigated the induction of PC in cells of the marine microalga *Phaeodactylum tricornutum* exposed to environmentally relevant levels of dissolved Cd, Cu and Pb by performing short-term incubations both in EDTA-buffered artificial seawater and in metal-enriched natural seawater samples. Our main objective was to

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examine whether accumulation of PC can be used as a biomarker of metal bioavailability in a bioassay for the assessment of metal pollution.

Materials and Methods

The unicellular marine diatom *P. tricornutum* Bohlin (Strain: CCAP 1052/1A) was obtained from the Culture Collection of Algae and Protozoa, Dunstaffnage Marine Laboratory, UK. Stock cultures were grown in axenic conditions, in natural seawater enriched with f/2 medium at one-fifth the normal trace metal concentration, at 21°C and fluorescent daylight (100 μmol photons m^{-2} s^{-1}) in a 16:8 light-dark cycle. Cell density was measured using a haemocytometer under a microscope. Prior to the metal incubation experiments, a preculture was prepared by inoculating algae from a stock culture on day 7 of growth (exponential phase) to provide an initial cell concentration of 5–7 × 10^4 cells mL^{-1} in natural seawater enriched with $NaNO_3$ and NaH_2PO_4 at a final concentration of 8.8 × 10^{-4} and 3.6 × 10^{-5} M, respectively (equivalent to N and P concentrations in f/2 medium). In this medium, the growth rate of *P. tricornutum* was similar (10%–15% lower) to that obtained in the maintenance medium. At the end of the logarithmic growth phase (approx. 2 × 10^6 cells mL^{-1}), calculated aliquots of the preculture were reduced to a volume of 10–20 mL by gentle filtration (1.2 μm membrane filters) and the resulting concentrated cell suspension was immediately transferred to the medium for PC induction experiments. We did not let the algae dry on the filter to avoid cell stress. Incubations were carried out both in EDTA-buffered artificial seawater and in natural seawater. Unless otherwise specified, we incubated 2 × 10^8 cells in 200 mL medium, to obtain a cell density of 1 × 10^6 cells mL^{-1}. PC induction experiments were carried out under continuous light conditions (100 μmol photons m^{-2} s^{-1}), at 21°C. Cell counts carried out after metal exposure showed that cell density was not appreciably changed during the incubation.

EDTA-buffered artificial seawater was prepared by following the Aquil recipe (Price et al. 1991) omitting the micronutrient metal stock solution, but adding EDTA (10.0 μM) and calculated amounts of Cd, Pb or Cu. Free metal ion concentrations in these metal buffers were calculated by means of the MINEQL+ chemical equilibrium program (Westall et al. 1976). All solutions were allowed to equilibrate overnight before performing tests.

Natural seawater was collected 3 miles offshore from the Island of Capraia (Tyrrhenian Sea, Italy), filtered through 0.45 μm membrane filters (Sartorius) and stored in the dark at +4°C. Before performing cell incubations, all the natural seawater samples were treated with $NaNO_3$ and NaH_2PO_4 at a final concentration equivalent to that of the preculture, to avoid that the cells undergo significant changes in the medium. Metal enriched natural seawater samples were prepared by adding increasing amounts of Cd in the range 5−100 nM and of Pb or Cu in the range 25−200 nM. After 16 h equilibration and before cell addition, the electrochemically labile fraction of dissolved metal ions (Me_{lab}) was measured, at natural pH (8.2), by Anodic Stripping Voltammetry. Seawater samples for field experiments were collected at six different stations, selected in contaminated areas in the province of the industrial city of Livorno, located in the Tyrrhenian coast of Tuscany (see map of Fig. 1). Tests were carried out by incubating 4 × 10^8 cells in a 1-L sample (4 × 10^5 cells mL^{-1}) for 6 h.

Metal-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 (diethylenetriaminepentacetic acid), then 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 (20,000*g*, 45 min) and the supernatant was used for the determination of thiols. Glutathione and phytochelatins were derivatized with the fluorescent tag monobromobimane (mBrB), by following the procedure reported by Rijstenbil and Wijnholds (1996) with some modifications, as described elsewhere (Morelli and Scarano 2001). Briefly, 200 μL of

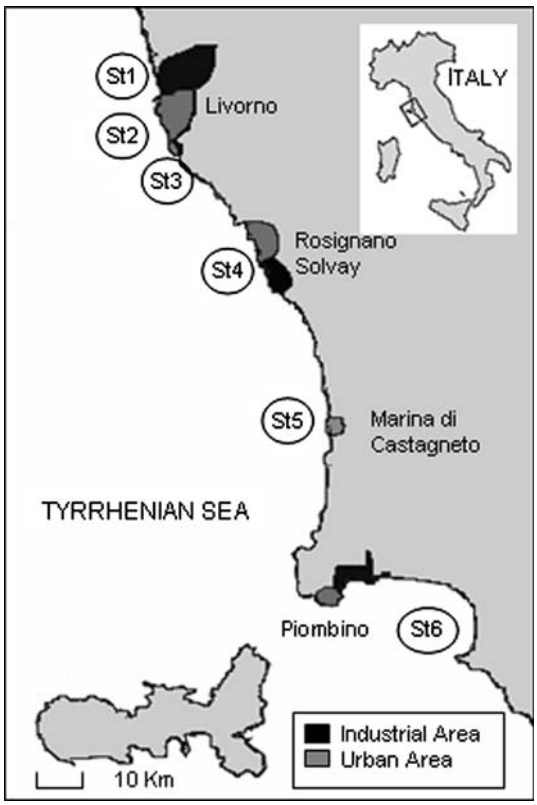

Fig. 1 Map of the province of Livorno where seawater samples were collected

the sample was added to 140 μL of 0.7 mM TCEP (Tris (2-carboxyethyl) phosphine) 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 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 methanesulfonic acid. The bimane derivatives were separated and quantified by high performance liquid chromatography (HPLC). 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 × 4.6 mm) and a fluorescence detector (RF–10AXL, Shimadzu). Retention time of phytochelatin oligomers was checked by using PC standards from *Silene vulgaris* (Friederich et al. 1998) kindly provided by Prof. Zenk, Munich University, Germany. Phytochelatins were quantified by using the relationship peak area versus concentration of GSH standard solutions. Total cellular PC concentration was expressed as the sum of the γ-Glu-Cys units quantified in each chromatographic peak of phytochelatins.

Voltammetric measurements were carried out by a Metrohm Model 646 VA processor in conjunction with a 647 VA Stand, equipped with a Metrohm multi-mode electrode (MME) used in the hanging mercury drop mode (HMDE). The measurements were carried out in differential pulse anodic stripping voltammetry (DPASV). The instrumental settings were: scan rate 12 mV s^{-1}, pulse duration 40 ms, pulse amplitude 50 mV, pulse repetition time 0.5 s, deposition time 300 s; deposition potential (E_{dep}) −1.2 V. The voltammetric sensitivities, obtained in seawater at pH = 2 spiked with $CdCl_2$ or $Cu(NO_3)_2$ or $Pb(NO_3)_2$ standard solutions, were 3.7×10^{-3}, 1.3×10^{-3} and 4.1×10^{-3} A M^{-1} s^{-1} for Cd, Cu and Pb, respectively. Determinations of heavy metals in seawater were performed at pH = 8.2 to measure the electrochemically labile fraction of the metal or at pH = 2 to measure the concentration of the total dissolved metal.

Results and Discussion

In order to evaluate the feasibility of using PC induction in *P. tricornutum* as a biomarker for metal exposure, 2×10^8 cells were incubated for 1 h in 200 mL of Aquil medium containing EDTA and Cd or Pb or Cu at the desired free-metal ion concentration. By taking into account the detection limit of our PC-assay protocol (1 pmol GSH injected, peak area 120), the incubation of 2×10^8 cells allows a reliable detection of 0.5 amol γ-Glu-Cys units $cell^{-1}$, equivalent to 3.0 pmol GSH (peak area, 360). The cellular PC concentration assayed in the cells before incubation was 0.3 ± 0.2 amol γ-Glu-Cys units $cell^{-1}$ (n = 3).

Under these exposure conditions, cadmium began to induce PC at a calculated free-Cd (Cd^{2+}) concentration of 0.2 nM (pCd^{2+} 9.7) corresponding to a value of total inorganic cadmium (Cd′) of 7.4 nM ($pCd' = pCd^{2+} + \log \alpha_{Cd}$, where $\alpha_{Cd} = Cd^{2+}/Cd' = 2.7 \times 10^{-2}$ is the coefficient calculated by MINEQL+ which corrects for inorganic side-reactions of cadmium in Aquil). Replicated 1-h exposures to Cd^{2+} in the range 0.1–50 nM ($10.0 > pCd^{2+} > 7.3$; $8.4 > pCd' > 5.7$) gave the dose–response curve pCd^{2+} versus total PC γ-Glu-Cys units reported in Fig. 2A. The lowest concentration of free-Pb (Pb^{2+}) inducing a measurable cellular PC induction was 0.6 nM (pPb^{2+} 9.2) corresponding to a value of total inorganic lead (Pb′) of 15.0 nM ($\alpha_{Pb} = Pb^{2+}/Pb' = 4.0 \times 10^{-2}$). Fig. 2B reports the dose–response curve for Pb^{2+} in the range 0.1–16 nM ($10.0 > pPb^{2+} > 7.8$; $8.6 > pPb' > 6.4$). At $pPb^{2+} = pCd^{2+} = 8.0$ cellular PC were about 15 and 40 amol γ-Glu-Cys units $cell^{-1}$, respectively. Copper began to induce PC at a calculated free-Cu concentration of 0.2 nM (pCu^{2+} 9.7) corresponding to

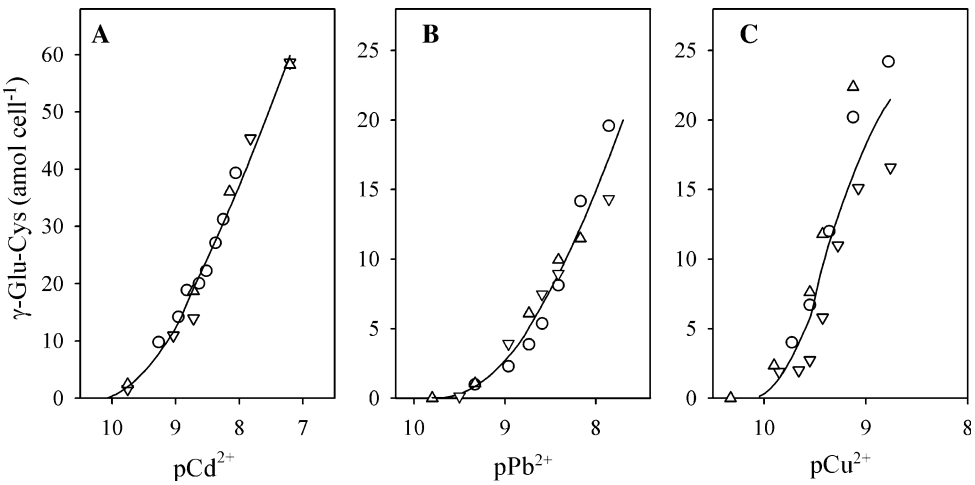

Fig. 2 Relationship between free metal ion concentration and phytochelatin synthesis in *P. tricornutum* after 1-h incubation in EDTA-buffered Aquil. Cell density, 1×10^6 cell mL^{-1}. Different symbols refer to three independent experiments. Phytochelatin concentration is expressed as the sum of the γ-Glu-Cys units in the individual oligomers

3.2 nM of total inorganic copper (Cu′) ($\alpha_{Cu} = Cu^{2+}/Cu' = 6.3 \times 10^{-2}$). Replicated 1-h exposures in the range 0.05–2.0 nM Cu^{2+} ($10.3 > pCu^{2+} > 8.7$; $8.8 > pCu' > 7.5$) gave the dose–response curve of Fig. 2C. At $pCu^{2+} = pPb^{2+} = pCd^{2+} = 9.0$ cellular PC was about 18, 2.5 and 13 amol γ-Glu-Cys units $cell^{-1}$, respectively, showing that, at this low concentration, Cu and Cd induced similar amounts of PC, whereas Pb was the weakest PC inductor. On the basis of the relationships reported in Fig. 2, it can be concluded that in a single metal exposure medium at fixed free metal ion concentration, the cellular pool of phytochelatins in the microalga *P. tricornutum* increases as the concentration of free metal ions Cd, Pb and Cu increases in solution, behaving as a biomarker of exposure to the bioavailable fraction of these metals in solution. These results agree with earlier studies (Ahner et al. 1995; Ahner and Morel 1995) carried out in a number of representative marine microalgae grown in steady-state conditions in EDTA-buffered Aquil, showing that the PC production is an increasing function of the free metal ion in the medium, even if the degree of PC synthesis varies widely among the species. *P. tricornutum* has been shown to be able to synthesize PC in response to the most common metals (Rijstenbil and Wijnholds 1996; Morelli and Scarano 2001; Ahner et al. 2002; Morelli and Scarano 2004), and its use in marine bioassays is widely diffused because of its easy cultivation and metal tolerance (Horvatić and Peršić 2007). The present results at low concentration of fixed free metal ions suggest that the presence of PC in this alga may represent an useful biological indicator for the assessment of metal pollution in marine waters.

Application of the bioassay to natural seawater was tested by examining the dose–response relationship between PC synthesis and metal concentration in natural seawater to which known amounts of Cd, Pb or Cu were added. Based on previous studies on the kinetics of PC synthesis in *P. tricornutum* (Morelli and Scarano 2001, 2004), which showed an increasing PC accumulation within few hours from Cd, Pb or Cu addition, a 6 h incubation time was used. The results (Fig. 3) show linear dose–response relationships in the ranges $4 \leq [Cd_{lab}] \leq 100$ nM, $20 \leq [Pb_{lab}] \leq 200$ nM, $10 \leq [Cu_{lab}] \leq 200$ nM, indicating that cellular PC accumulation increases as a function of the labile metal concentration initially present in seawater. Since this operationally defined metal fraction comprises both inorganic and electrochemically dissociable metal organic complexes (Achterberg and Braungardt 1999), our results demonstrate the suitability of PC as a biomarker of exposure to the bioavailable forms of the metal dissolved in natural seawater, in agreement with our findings in media at fixed free metal ion concentration. In natural seawater, the absence of a metal buffer hinders the relationship between PC and pMe^{2+} to be obtained, thus a comparison with the EDTA-buffered artificial seawater cannot be accomplished. In fact, cell-mediated processes, such as the adsorption on cell surfaces and/or the release of extracellular organic ligands able to form biologically inert complexes with the metal ions, can affect the concentration of the metal ion in solution, lowering the PC response. So, it should be expected that, in natural seawater, lower cell densities can enhance the PC response. As a matter of fact, an increase of about 25% of the cellular PC concentration was assayed in cells in response to copper from 20 to 76 nM when tests were carried out incubating cells at a concentration of 1×10^4, rather than of 1×10^6 cells mL^{-1} (data not reported).

As an application of the bioassay to polluted natural seawater, 6-h incubation tests were carried out on six different samples, collected along the coast in the province of Livorno, as shown in the map of Fig. 1. The results show that these waters were able to induce PC synthesis enhancing the cellular pool with concentrations ranging from 7.0 to 10.1 amol γ-Glu-Cys units $cell^{-1}$ (Table 1).

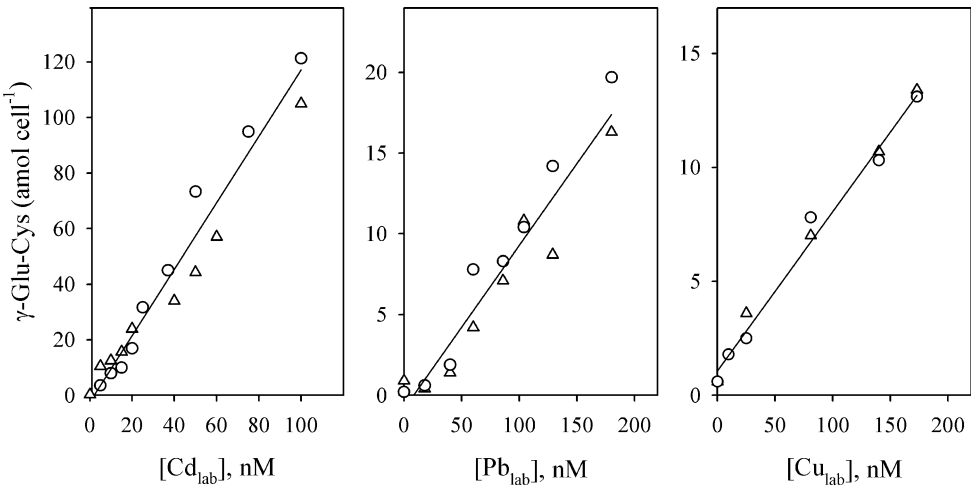

Fig. 3 Relationship between electrochemically labile metal concentration in seawater and phytochelatin synthesis in *P. tricornutum* after 6-h incubation in metal – enriched natural seawater. Cell density, 1×10^6 cell mL^{-1}. Different symbols refer to two independent experiments. Phytochelatin concentration is expressed as the sum of the γ-Glu-Cys units in the individual oligomers

Table 1 Total dissolved metal concentrations (nM) in the sampling stations (map in Fig. 1) and phytochelatin concentration in cells of *P. tricornutum* after 6-h incubation in the seawater samples[a]

Site	Cd	Pb	Cu	Zn	PC[b]
Station 1	0.4 ± 0.1	0.9 ± 0.2	60 ± 11	80 ± 10	8.2 ± 1.5
Station 2	0.1 ± 0.1	1.6 ± 0.4	54 ± 7	123 ± 12	7.0 ± 0.3
Station 3	0.3 ± 0.2	0.9 ± 0.3	38 ± 5	61 ± 8	7.0 ± 0.6
Station 4	0.4 ± 0.3	0.8 ± 0.2	40 ± 6	88 ± 11	8.2 ± 1.7
Station 5	0.2 ± 0.1	1.5 ± 0.5	36 ± 4	72 ± 8	7.3 ± 1.4
Station 6	9.3 ± 0.3	1.4 ± 0.1	36 ± 7	102 ± 6	10.1 ± 0.4
EDTA-SS[c]	–	–	–	–	0.5 ± 0.4[d]
Control seawater	0.1 ± 0.1	0.7 ± 0.1	7 ± 2	15 ± 2	0.3 ± 0.2

[a] Cell density, 4×10^5 cell mL^{-1}

[b] PC are expressed as γ-Glu-Cys units (amol $cell^{-1}$). The results refer to three independent experiments

[c] EDTA-treated (10 μM) seawater samples

[d] $n = 6$; Metal concentrations are mean values ± S.D. of two to three measurements carried out at pH = 2 by ASV

Incubation tests were then repeated on the same samples previously treated with EDTA (10 μM) and left to stand overnight. Incubations in such EDTA-treated waters produced no enhanced PC synthesis relative to cells before incubation (0.5 ± 0.4 amol γ-Glu-Cys units $cell^{-1}$). Since EDTA is a strong metal ion complexing agent, this result shows that the PC-inducing species in these waters must be metal ions in a bioavailable form, that are converted by EDTA in biologically inert complexes.

The PC synthesis induced by such samples must be considered a cumulative response to all metal ions able to induce PC in this microalga. Because a complete analysis of metals in these waters was not performed, we can only speculatively attribute the cellular response on the basis of ASV measurement of the more common PC-inducing metal ions. ASV assays, at pH = 2, revealed the concentrations of total Zn, Cd, Pb, and Cu reported in Table 1. Cd and Pb levels were similar to those assayed in the control seawater (Island of Capraia, Tyrrhenian Sea), except in station 6, in which Cd was one order of magnitude higher. In all the assayed samples, Zn and Cu were higher than in the control seawater. For as regards Zn, it has been reported (Morelli and Scarano 2001; Kawakami et al. 2006a) that cells of *P. tricornutum* exposed to Zn at 1 μM did not exhibit PC synthesis in short-term experiments, so the hypothesis could be made that copper is mainly responsible for the response. It has been reported (Wei et al. 2003; Le Faucheur et al. 2005) that the PC production can be not only related to the exposure to a single metal, but can be the result of antagonistic and synergistic effects of multiple metals. According to Kawakami et al. (2006a), Zn did not affect the amount of PC induced in *P. tricornutum* cells by Cu, whereas it had an antagonistic effect on the induction of PC by Cd. So, the inability of Zn to affect the PC response to Cu in *P. tricornutum* supports the hypothesis that the induction of PC in this alga incubated in our seawater samples was triggered by copper, although a contribution of Cd in station 6, which exhibited the highest PC response, cannot be excluded. In coastal seawater, copper is almost entirely complexed by organic ligands (Buck et al. 2007), but it is possible that in areas impacted by anthropogenic and industrial inputs the copper concentration exceeds that of the organic ligands, resulting in high levels of free Cu^{2+}. In our metal-polluted seawater samples the exact speciation of the metal ions cannot be ascertained, nevertheless the ability of these waters to induce PC means that a fraction of the total dissolved metals (mainly copper) was in a bioavailable form, potentially toxic, at a concentration sufficient to activate a cellular detoxification system. Although many studies have reported that several planktonic marine and freshwater algae are able to synthesize PC in response to toxic heavy metals (Kawakami et al. 2006b) only few attempts have been made to use phytochelatins as biomarkers in a metal – specific bioassay (Wei et al. 2003; Le Faucheur et al. 2005). The response of the marine microalga *P. tricornutum* suggests that these cellular peptides could represent a useful biomarker for the assessment of marine and freshwater toxicity, as well as for the assessment of sediment toxicity by carrying out PC-induction tests on sediment elutriate. Further studies on other species of phytoplanktonic algae and more systematic investigations in the field are in progress in our lab to validate the use of bioassays based on phytochelatin synthesis.

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