BioMetals (2006) 19:51–60
DOI 10.1007/s10534-005-5115-6
© Springer 2006

Effects of metal combinations on the production of phytochelatins and glutathione by the marine diatom *Phaeodactylum tricornutum*

Silvia K. Kawakami[1], Martha Gledhill[2] & Eric P. Achterberg[2,*]
[1]*School of Earth, Ocean and Environmental Science, University of Plymouth, Plymouth, PL4 8AA, UK;* [2]*School of Ocean and Earth Science, University of Southampton, National Oceanography Centre Southampton, Southampton, SO14 3ZH, UK; *Author for correspondence (Tel: +44-2380593199; Fax: +44-2380593059; E-mail: eric@soc.soton.ac.uk)*

Received 21 February 2005; accepted 06 April 2005

Key words: glutathione, metal toxicity, metal stress, *Phaeodactylum tricornutum*, phytochelatins, thiols

Abstract

Copper, Cd and Zn can be found at elevated concentrations in contaminated estuarine and coastal waters and have potential toxic effects on phytoplankton species. In this study, the effects of these metals on the intracellular production of the polypeptides phytochelatin and glutathione by the marine diatom *Phaeodactylum tricornutum* were examined in laboratory cultures. Single additions of Cu and Cd (0.4 μM Cu^{2+} and 0.45 μM Cd^{2+}) to the culture medium induced the production of short-chained phytochelatins ($(\gamma$-Glu-Cys$)_n$-Gly where n = 2–5), whereas a single addition of Zn (2.2 μM Zn^{2+}) did not stimulate phytochelatin production. Combination of Zn with Cu resulted in a similar phytochelatin production compared with a single Cu addition. The simultaneous exposure to Zn and Cd led to an antagonistic effect on phytochelatin production, which was probably caused by metal competition for cellular binding sites. Glutathione concentrations were affected only upon exposure to Cd (85% increase) or the combination of Cd with Zn (65% decrease), relative to the control experiment. Ratios of phytochelatins to glutathione indicated a pronounced metal stress in response to exposures to Cu or Cd combined with Zn. This study indicates that variabilities in phytochelatin and glutathione production in the field can be explained in part by metal competition for cellular binding sites.

Introduction

Phytochelatins (PCs) and glutathione (GSH) are metal-binding and thiol (-SH or sulfhydryl group) containing peptides synthesized by eukaryotes for various cellular functions, notably protection against oxidative stress, metal detoxification and homeostasis (Grill *et al.* 1985; Cobbett 2000). These peptides contain three different amino acids: glutamate (Glu), cysteine (Cys) and glycine (Gly), with the basic formula $(\gamma$-Glu-Cys$)_n$-Gly, with n = 1 for GSH and n = 2–11 for PCs (Grill *et al.* 1985). The concentrations of the peptides, particularly PCs, in phytoplankton cells increase when organisms are exposed to enhanced concentrations of a wide range of metals (Grill *et al.* 1985). The enzyme phytochelatin synthase catalyses the phytochelatin production *in vitro*, and requires a metal ion for activity (Grill *et al.* 1985). Phytochelatin synthase catalyzes the transfer of γ-Glu-Cys from GSH to another GSH molecule to form PC2, or to $(\gamma$-Glu-Cys$)_n$-Gly to form PC chain lengths of n + 1. Phytochelatin production serves to detoxify intracellular metals by chelation through coordination with the sulfhydryl group in cysteine; a mechanism that is analogous to the operation of metallothioneins in plant, fungal, animal and cyanobacterial cells (Rauser 1990). The production of GSH and PCs is dependent upon the phytoplankton species, the degree of toxicity of the metal ions and interactions among metals (Ahner & Morel 1995; Ahner *et al.* 1995; Knauer *et al.* 1998; Wei *et al.* 2003).

Furthermore, these workers have shown that thiol production is related to aqueous free metal ion concentrations, rather than the total dissolved metal concentrations. Experiments using phytoplankton monocultures under stress of one single metal form a valuable tool to assess the primary implications and effects of a toxic metal on phytoplankton cells. However, such single addition experiments provide a limited scenario for the interpretation of field observations. Little is known about the effects of mixtures of metals on phytochelatin and glutathione production by marine phytoplankton.

The toxic effects of high concentrations of metals on the marine diatom *Phaeodactylum tricornutum* in laboratory experiments include decrease in growth rate, photosynthesis rate, chlorophyll *a* content and intracellular ATP concentration (Cid *et al*. 1995; Torres *et al*. 2000). However, no direct evidence has been documented of metal toxicity to phytoplankton assemblages, or *P. tricornutum* in particular, in the field. The determination of intracellular thiols therefore forms an alternative way to assess metal stress in phytoplankton. *Phaeodactylum tricornutum* is an efficient producer of phytochelatins, capable of readily synthesising a wide range of phytochelatins under metal stress (Rijstenbil & Wijnholds 1996). Several studies have been undertaken to characterise phytochelatins produced by *P. tricornutum* (Morelli & Scarano 1995; Rijstenbil & Wijnholds 1996; Morelli & Pratesi 1997; Torres *et al*. 1997; Scarano & Morelli 2002). The effects of combinations of toxic metals on phytochelatin and glutathione production by *P. tricornutum*, however, have not yet been investigated. The aim of this study was to undertake short-term metal exposure experiments using *P. tricornutum* cultures in order to investigate the effects of single additions of Cu, Zn and Cd and combinations of these metals on phytochelatin and glutathione production. The experiments were undertaken using free ionic metal concentrations that are relevant to contaminated estuarine and coastal waters.

Materials and methods

Reagents and laboratory ware

Glassware and plastic bottles were soaked in 1 M HCl (Aristar, VWR Ltd) for at least 24 h to remove metal contamination, and then rinsed three times with de-ionized water (>18.2 MΩ cm^{-1}; MilliQ, Millipore). Reagents and solvents were HPLC or Aristar grade, unless otherwise stated. Glutathione standard (98%, Sigma Ltd) and phytochelatin 2 (dimer PC2: γ-Glu-Cys)$_2$-Gly; Pepsin Co., UK), were prepared at concentrations of 0.01 M and 10–100 μM, respectively, in a solution containing 0.1 M HCl and 5 mM diethylenetriamine pentaacetic acid (DTPA, 99%, Sigma Ltd).

Culture medium

The culture medium was a synthetic ocean water (SOW) based on the Aquil recipe (Morel *et al*. 1979; Price *et al*. 1988). A stock solution of 20 l of the SOW containing only the major salts (Analar grade, VWR Ltd) was prepared using de-ionised water. When required, the stock solution was transferred to 1.17 l polycarbonate bottles (Nalgene) and subjected to microwave treatment (1 min, 900 W) to sterilise the stock solution and bottles prior to use. Subsequently, filter-sterilised (using a Teflon 0.2 μm pore size membrane syringe filter; Sartorius Ltd) trace metal, nutrients, vitamin and ethylenediaminetetraacetate (EDTA; VWR Ltd) solutions were added to yield final concentrations according to the Aquil recipe, and the medium was allowed to equilibrate overnight prior to seeding with diatoms. The concentrations of the components in the culture medium are presented in Table 1; the pH of the medium was 8.1.

Stock culture of Phaeodactylum tricornutum

Cells of the diatoms *P. tricornutum* Bohlin were obtained from the collection of the Marine Biological Association (Plymouth, UK). Stock cultures of *P. tricornutum* were grown in the Aquil culture medium (salinity of the SOW = 17), at 15 °C under continuous light conditions, until the late exponential growth phase and a cell density of about 8.0×10^8 cells l^{-1}. The culture was manually shaken once a day. Cells were counted daily using a microscope (Olympus) and an Improved Neubauer haemocytometer.

Short-term metal exposure experiments

The conditions of the metal exposure experiments and the concentrations of metals employed in this

Table 1. Composition of the synthetic ocean water based on the Aquil recipe and used as a culture medium for the phytoplankton stock culture and the short-term metal exposure experiments.

Substances	Concentration
Major salts	
NaCl	2.1×10^{-1} M
Na_2SO_4	1.5×10^{-2} M
KCl	4.7×10^{-3} M
$NaHCO_3$	1.4×10^{-3} M
$MgCl_2{\cdot}6H_2O$	2.8×10^{-2} M
$CaCl_2{\cdot}2H_2O$	0.6×10^{-2}
Microelements	
KBr	4.2×10^{-4} M
KI	2.4×10^{-5} M
H_3BO_3	2.5×10^{-4} M
NaF	3.6×10^{-5} M
$SrCl_2{\cdot}6H_2O$	3.2×10^{-5} M
Nutrients	
$NaH_2PO_4{\cdot}H_2O$	2.0×10^{-5} M
$NaNO_3$	2.0×10^{-4} M
$Na_2SiO_3{\cdot}9H_2O$	1.0×10^{-4} M
Trace metals	
$FeCl_3{\cdot}6H_2O$	0.5×10^{-6} M
$ZnSO_4{\cdot}7H_2O$	2.3×10^{-8} M
$MnCl_2{\cdot}4H_2O$	9.1×10^{-8} M
$CoCl_2{\cdot}6H_2O$	2.6×10^{-8} M
$CuSO_4{\cdot}5H_2O$	0.5×10^{-8} M
$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$	10.4×10^{-8} M
Na_2SeO_3	1.0×10^{-8} M
Chelating agent	
Na_2EDTA	5.0×10^{-6} M
Vitamins	
B_{12}	5.5×10^{-7} g l^{-1}
Biotin	5.0×10^{-7} g l^{-1}
Thiamine HCl	1.0×10^{-4} g l^{-1}

study were based on Rijstenbil & Wijnholds (1996) and Morelli & Scarano (2001). The experiments were performed in duplicate. Aliquots of 200 ml of the stock culture (at late exponential growth phase) were transferred to 1.17 l polycarbonate bottles containing 600 ml of the Aquil culture medium (salinity 17; total volume 800 ml), to allow a continuous cell growth for the short-term metal exposure experiments. The cultures were spiked with metal solutions, except the control experiment, as follows: (1) control – no addition of metals; (2) 10 μM $Cd(NO_3)_2$; (3) 10 μM $Cu(NO_3)_2$; (4) 10 μM $Zn(NO_3)_2$; (5) 10 μM $Cu(NO_3)_2$ + 10 μM $Zn(NO_3)_2$; (6) 10 μM $Cd(NO_3)_2$ + 10 μM $Zn(NO_3)_2$. The incubations were conducted under continuous light conditions at 15 °C for 24 h. After this period, the cultures were filtered and prepared for the analysis of phytochelatins and glutathione. Free aqueous metal concentrations were calculated from the total metal, major ion and EDTA concentrations in the culture medium using the thermodynamic speciation programme MINEQL + (Schecher & Mcavoy 1992).

Analysis of phytochelatins and glutathione produced by P. tricornutum

The analysis of thiols produced by the metal-treated diatom cultures was based on the method reported by Rijstenbil & Wijnholds (1996). Briefly, 500 ml of culture solution was filtered through a 0.45 μm nitrocellulose membrane filter (Sartorius Ltd), using gentle vacuum to avoid cell breakage. The filters were placed in a microtube (1.5 ml; Eppendorf) and the extraction of thiols from the phytoplankton cells was carried out at 0 °C by ultrasonication following addition of 1.2 ml solution of 0.1 M HCl and 5 mM DTPA. The extraction was followed by centrifugation at high speed (1300 g/20 min at 4 °C). Aliquots of 250 μl algal extracts were treated with 25 μl of a 20 mM solution of the reductant tris (2-carboxyethyl) phosphine hydrochloride (Sigma Ltd) in 0.1 M HCl containing 5 mM DTPA, for 5 min. Then 160 μl 200 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethansulphonic acid (HEPES)/5 mM DTPA at pH 9.0 was added. After another 5 min, 10 μl 100 mM of the fluorescent tag monobromobimane (Fluka Ltd) and 465 μl HEPES/DTPA were added. The reaction was stopped by addition of 100 μl 1 M methanesulfonic acid (99%, Fluka) and the samples were stored in the dark at 4 °C until HPLC analysis.

HPLC analysis

The HPLC system consisted of two pumps (Merck-Hitachi Models L-6200 and L-6000), a Rheodyne injection valve with a 20 μl loop and a Model FD-300 fluorescence detector (Dionex) operating at 380 nm (excitation) and 470 nm (emission) wavelengths. Separation of the thiols was carried out using a 150 × 4.6 mm C-18

(Econosphere) HPLC column with 3 μm particle size. Solvent A was 0.1% trifluoroacetic acid (in de-ionised water) and solvent B was acetonitrile. The flow rate of the solvent was 1.0 ml min^{-1}. The gradient, adapted from Rijstenbil & Wijnholds (1996) was: 0–13 min, 10–21% B; 13–33 min, 21–35% B; 33–40 min, 35–100% B; 40–50 min, isocratic 100% B; 50–65 min, 100–10% B.

Results

Experimental conditions

The concentrations of the various metal fractions (including free aqueous metals, inorganic metals and EDTA complexed metals) in the culture media containing EDTA (5×10^{-6} M) are presented in Table 2. The chelating agent EDTA forms metal complexes that are not taken up by phytoplankton, thus serving to control metal speciation and bioavailability in the medium (Price *et al.* 1988). The inorganic metal fraction includes kinetically labile species such as the hydroxide, chloride and carbonate metal complexes as well as the free aqueous metal ions. In a medium with constant pH and inorganic ligand concentrations (such as seawater), the inorganic and free aqueous metal concentrations are strictly proportional.

At metal concentrations used in this study, no decrease in growth was observed, as the cell numbers in the metal-treated and control experiments did not show significant differences after the 24 h incubation (< 2% difference; Table 2). In contrast, Rijstenbil & Wijnholds (1996) reported a 30% decrease in growth rate of *P. tricornutum* at concentrations of free aqueous Cu^{2+} and Cd^{2+} similar to those used in our experiments.

Typical examples of calibration graphs for GSH and PC2 are presented in Figure 1. The fluorescent responses to PC2 were approximately double compared to GSH. Therefore, the quantification of phytochelatins PC3, PC4 and PC5 produced in the short-term metal exposure experiments was based on the PC2 calibration, using the assumption that the fluorescence response for the polypeptides was proportional to the number of SH groups (Ahner *et al.* 1995; Rijstenbil & Wijnholds 1996). The reproducibility of the short-term metal exposure experiments was assessed using Cd at a free ionic concentration of 0.45 μM, for five replicates. Relative standard deviations (RSD) between 16% and 24% were observed for the chromatographic peak areas of GSH and PC2, PC3, PC4 and 30% for PC5. These results show that the incubation experiments resulted in a reproducible thiol production.

Effects of metal additions on phytochelatin and glutathione production by Phaeodactylum tricornutum

The concentrations of PCs in *P. tricornutum* were normalized to cell numbers (amol

Table 2. Concentrations (in M) of the major EDTA complexes and free ionic metal (M^{2+}) and inorganic metal species (M′) in the experiments calculated using the MINEQL+ programme.

Species (M)	Exposure experiment					
	Control	Zn	Cu	Cd	Cu + Zn	Cd + Zn
[ZnEDTA]	2.3×10^{-8}	4.6×10^{-6}	3.7×10^{-10}	1.8×10^{-8}	1.5×10^{-7}	3.5×10^{-6}
Zn′	2.4×10^{-10}	5.4×10^{-6}	2.3×10^{-8}	5.4×10^{-9}	9.9×10^{-6}	6.5×10^{-6}
Zn^{2+}	9.8×10^{-11}	2.2×10^{-6}	9.4×10^{-9}	2.2×10^{-9}	4.1×10^{-6}	2.7×10^{-6}
[CuEDTA]	5.0×10^{-9}	4.9×10^{-9}	5.0×10^{-6}	5.0×10^{-9}	4.8×10^{-6}	4.9×10^{-9}
Cu′	8.7×10^{-13}	9.2×10^{-11}	5.0×10^{-6}	2.5×10^{-11}	5.1×10^{-6}	1.4×10^{-10}
Cu^{2+}	6.8×10^{-14}	7.5×10^{-12}	4.0×10^{-7}	2.0×10^{-12}	4.1×10^{-7}	1.1×10^{-11}
[CdEDTA]	–	–	–	4.4×10^{-6}	–	1.2×10^{-6}
Cd′	–	–	–	5.6×10^{-6}	–	8.9×10^{-6}
Cd^{2+}	–	–	–	4.5×10^{-7}	–	7.7×10^{-7}
Cells l^{-1} before incubation	2.00×10^{8}	1.92×10^{8}	1.58×10^{8}	1.92×10^{8}	1.25×10^{8}	2.70×10^{8}
Cells l^{-1} after incubation	2.00×10^{8}	1.92×10^{8}	1.58×10^{8}	1.92×10^{8}	1.25×10^{8}	2.75×10^{8}

Improved log *K* values were inserted into the MINEQL+ database from the National Institute of Standards Technology (NIST) database for the following compounds: $MgHEDTA^{-}$, ZnHEDTA, $CuEDTA^{2-}$, $EDTAH^{3-}$, $EDTAH_2^{2-}$, $EDTAH_4$, EDTA H_5, $EDTAH_3^{-}$, $CaEDTA^{2-}$, CdEDTA, $Zn(OH)_3^{-}$, $ZnCl_2$ aq, $Cu_2(OH)_2^{2}$, $Cu(OH)_3^{-}$, $Cu(OH)_2$aq (Twiss *et al.* 2001; Serkiz *et al.* 1996). Cell numbers were counted before and after the incubation period.

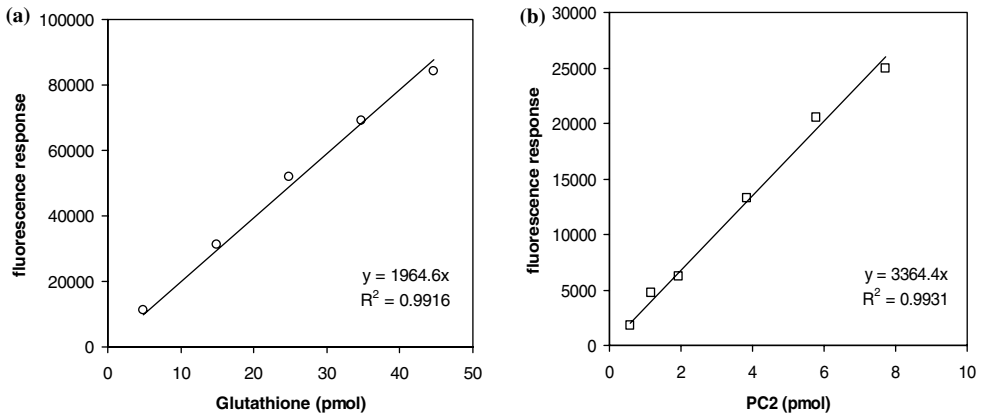

Figure 1. Calibration graphs for: (a) glutathione; (b) phytochelatin PC2.

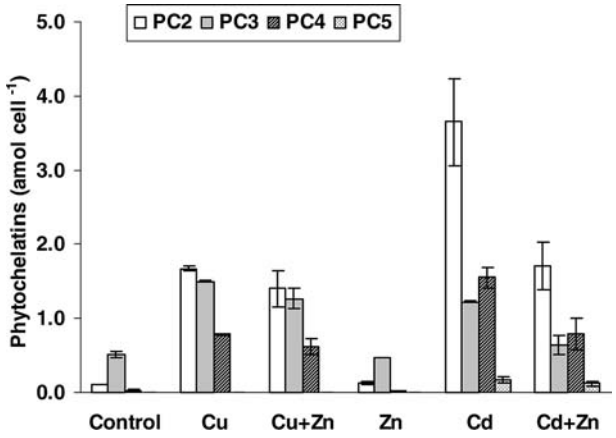

Figure 2. Phytochelatins produced by *P. tricornutum* under metal exposure. Bars span the range from the average for duplicate analyses.

cell$^{-1} = 10^{18}$mol cell^{-1}). The production of PCs with *n* ranging from 2 to 5 (PC2–PC5) was induced in all metal exposure experiments, with concentrations ranging from 0.1 to 3.6 amol cell^{-1} (Figure 2). The control showed low concentrations of PCs, which most likely indicate basal levels of intracellular PCs. Exposure to Zn did not induce phytochelatin production in *P. tricornutum*, and showed similar results to the control. Exposure to Cd only resulted in a more pronounced PC production compared to exposure to Cu only. The Cd + Zn incubation resulted in a reduced PC production compared with Cd only. Whereas, the exposure to Cu + Zn resulted in similar PC concentrations relative to the induction observed by the addition of Cu only. When excess PCs were produced the dimer PC2 was the most abundant polypeptide, for example, in the Cd and Cd + Zn exposures the concentrations of PC2 were at least double that of other PCs.

In the incubation experiments the concentrations of the thiol GSH in *P. tricornutum* ranged from 4.7 to 25.3 amol cell^{-1} (Figure 3). Glutathione was also present in phytoplankton cells in the control experiments, which can be explained by its role in a range of biochemical intracellular functions including the maintenance of reducing conditions for amino acids and proteins, and the protection against oxidative and radiation damage (Ahner *et al.* 2002). Glutathione occurred at similar concentrations in all exposure experiments, except for incubations with added Cd only and with Cd in combination with Zn. These metal treatments caused an 85% increase and 65%

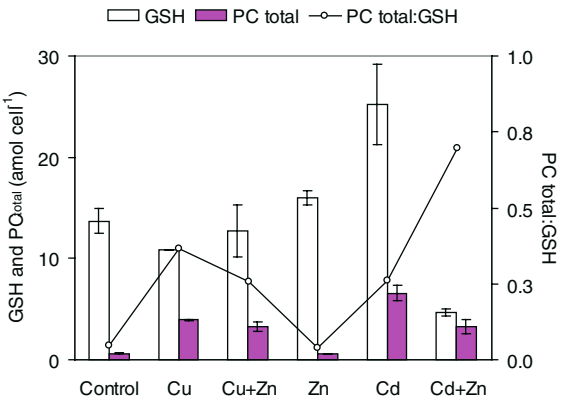

Figure 3. Glutathione produced by *P. tricornutum* under metal exposure, and PC_{total}: GSH ratios. Bars span the range from the average for analyses of duplicate incubation experiments.

depletion in GSH concentration, respectively, relative to the control.

PC_{total}: GSH ratios

The ratios of the concentrations of PC_{total} to GSH, in which PC_{total} corresponds to the sum of the γ-Glu-Cys units of the PC oligomers, have been suggested to provide an indication of the extent of metal stress (Tang *et al.* 2000). In the present experiments, the ratios ranged from 0.11 to 1.95 (Figure 3). The lowest PC_{total}: GSH ratio was observed for the control experiment and the highest ratio for the combination of Cd + Zn.

Discussion

The application of the Aquil culture medium, which is chemically well-defined and metal buffered, allowed us to investigate the influence of metal exposure by single metals and metal combinations on thiol production in *P. tricornutum*. Phytochelatin production in *P. tricornutum* is an important detoxification mechanism against metals that have entered the cell. *P. tricornutum* produced a range of PC compounds, in response to metal exposures. In addition, changes in intracellular GSH concentrations were observed for different exposure conditions. A comparison of PC and GSH concentrations reported by different workers for *P. tricornutum* under different metal exposure conditions, together with PC_{total}: GSH ratios is presented in Table 3.

Phytochelatin production is thus related to aqueous free metal ion concentrations, which determine the cellular metal quota through uptake kinetics. In the present study using *P. tricornutum*, Cd was the most effective inducer of PC2 which is in agreement with previous studies including those using other phytoplankton species (Ahner & Morel 1995; Ahner *et al.* 1995; Lee *et al.* 1996). Cadmium is one of the most toxic trace metals and is found in high concentrations in discharges from zinc smelters, electroplating industry and sewage treatment plants. Lee *et al.* (1996) hypothesised that the diatom *Thalassiosira weissflogii* exports the Cd–PC complex as a metal detoxification mechanism. These workers observed that once outside the cell, the Cd–PC complex appeared not to be stable as the exported Cd was again able to induce PC synthesis. It is not known whether *P. tricornutum* utilises a similar Cd–PC export mechanism.

Copper exposure resulted in a less pronounced PC production by *P. tricornutum* compared with Cd. This may be due to a higher toxicity of Cd compared to Cu, or alternatively to a higher intracellular Cd concentration. Ahner *et al.* (2002) reported a greater PC production response to Cu rather than Cd for *P. tricornutum*. These contrasting observations indicate that the cellular Cd and Cu quota were different compared to our experiments, or that there were important physiological differences between laboratory strains of the same phytoplankton species.

Our results indicated that the short-term exposure experiments involving Zn only, produced no enhanced PC production relative to the control (see Figures 2 and 3). Zinc is able to activate PC synthase in vitro (Grill *et al.* 1985) and *in vivo* (Ahner & Morel 1995), but is reported as being a weak inducer of PC production in algae, plants and fungi (Morelli & Scarano 2001 and references therein). Deleterious effects of Zn on other physiological parameters than PC production have not been reported for *P. tricornutum*. It is likely that Zn is accumulated in cellular compartments such as the skeleton, where it would have few negative effects on cell functioning.

In our experiments, we observed that Zn had an antagonistic effect on the induction of PCs by Cd. Zinc forms stronger complexes with organic ligands in the oxygenated extracellular environment of the phytoplankton cells compared with Cd

Table 3. Production of PC_{total} (sum of γ-Glu-Cys units of the oligomers) and GSH, together with thiol ratios for laboratory *P. tricornutum* cultures under metal stress.

Metal exposure experiment	Metal concentration/ M	GSH	PC_{total}	PC_{total}: GSH	Comments	Reference
Control	$[Cu^{2+}] = 6.8 \times 10^{-14}$	13.7	1.81	0.13	PCs and GSH concentrations are given as γ-Glu-Cys amol cell^{-1}	This study
	$[Zn^{2+}] = 9.8 \times 10^{-11}$ [Cd^{2+}] = not added as micronutrient					
Cu	$[Cu^{2+}] = 4.0 \times 10^{-7}$	10.9	11.0	1.01	Salinity = 17; 24 h incubation	
Cu + Zn	$[Cu^{2+}] = 4.1 \times 10^{-7}$ $[Zn^{2+}] = 4.1 \times 10^{-6}$	12.8	9.09	0.71		
Zn	$[Zn^{2+}] = 2.2 \times 10^{-6}$	16.0	1.73	0.11		
Cd	$[Cd^{2+}] = 4.5 \times 10^{-7}$	25.3	18.0	0.71		
Cd + Zn	$[Cd^{2+}] = 7.7 \times 10^{-7}$ $[Zn^{2+}] = 2.7 \times 10^{-6}$	4.68	9.12	1.95		
Control		780	14.6	0.019	PCs and GSH concentrations are given as μM (μmol cell volume^{-1})	Ahner *et al.* (2002)
Cd	$[Cd^{2+}] = 10^{-11}$	560	2.90	0.052		
Cd	$[Cd^{2+}] = 10^{-10}$	680	53.4	0.079	24 h incubation; salinity = 35	
Cd	$[Cd^{2+}] = 10^{-9}$	710	51.6	0.073		
Cu	$[Cu^{2+}] = 10^{-11}$	260	81.1	0.31		
Cu	$[Cu^{2+}] = 10^{-10}$	860	116	0.14		
Cu	$[Cu^{2+}] = 10^{-9}$	2100	247	0.12		
Control			–	–	PCs and GSH concentrations are given as γ-Glu-Cys amol cell^{-1} (obtained from graphs)	Morelli and Scarano (2001)
Cd	$[Cd_{Total}] = 10 \times 10^{-6}$	40	65	1.6		
Pb	$[Pb_{Total}] = 10 \times 10^{-6}$	45	80	1.8	Salinity = 35; 7 h incubation	
Zn	$[Zn_{Total}] = 10 \times 10^{-6}$		–	–		
Control		3385	79	0.023	PCs and GSH concentrations are in SH μM (biovolume)	Rijstenbil and Wijnholds (1996)
Cu	$[Cu] = 0.4 \times 10^{-6}$	2359	379	0.16		
Cd	$[Cd] = 9.0 \times 10^{-6}$	2173	1424	0.66	Salinity = 17; 24 h incubation	

according to the Irving–Williams order of affinity (da Silva & Williams 1991). This may result in the replacement of Cd by Zn ions on metal uptake sites of *P. tricornutum* with a consequent reduction in PC production. A similar decrease in PC production by Cd in diatoms at enhanced Zn and Mn concentrations has been observed by Wei *et al.* (2003). The authors explained these observations by competition between these metals for uptake sites. Furthermore, Sunda & Huntsman (1996) showed that Cd uptake by *Thalassiosira pseudonana* takes place through the same channels as Zn

and Mn, thus Zn and Mn may competitively inhibit Cd toxicity and the induction of PCs by Cd. In contrast, the presence of Zn did not have any effect on the production of PCs and GSH by Cu indicating that no competitive interaction between these metals occurred with respect to thiol production. This observation is in line with the ability of Cu to form stronger complexes with organic ligands in the extracellular environment of the cells, compared with Zn (da Silva & Williams, 1991), and hence Zn would not be able to outcompete Cu at the uptake sites. Alternatively, the absence of an influence of Zn on thiol production by Cu in *P. tricornutum* may be the result of the presence of two different transport systems for Zn and Cu, which has been reported for higher plants (Fox & Guerinot 1998; Williams *et al.* 2000).

The production of PCs by phytoplankton is reported to occur almost immediately upon metal exposure, and a steady state concentration is typically reached within 1 day. Ahner *et al.* (2002) reported that a culture of *Emiliania huxleyi* in the exponential phase reached a steady state concentration for phytochelatin within 10 h following either Cd ($Cd^{2+} = 10^{-9}$M) or Cu ($Cu^{2+} = 10^{-9}$M) exposures, whereas an exponential culture of *T. pseudonana* had not yet reached a steady state PC concentration after 36 h following Cd ($Cd^{2+} = 10^{-9}$M) exposure. Morelli & Scarano (2001) reported that the synthesis of PCs in *P. tricornutum* commenced within ca. 15 min of the initiation of the exposures to either 10 μM Cd or 10 μM Pb (total concentrations). These workers observed PC concentrations after 7 h of incubation (approximately 6 amol cell^{-1} for each of the oligomers $n = 2$, 3 and 4; calculated from graph), that are in agreement with values observed in the present study. Such a rapid formation of PCs in *P. tricornutum* cells, together with their capability to release GSH-like substances and surfactants (Croot *et al.* 2000) could account for the high metal tolerance of this diatom species (EC_{50} value for total dissolved Cd is 200 μM; Torres *et al.* 1997). In addition, a recent study reported that *P. tricornutum* cells can respond rapidly to metal-induced oxidative stress by activation of a suite of antioxidant enzymes (Morelli & Scarano 2004). In contrast, metal sensitive phytoplankton species such as *Skeletonema costatum* show growth inhibition at 100 times lower Cd concentrations (Torres *et al.* 1997). This species has also shown a less pronounced PC production than *P. tricornutum* (Rijstenbil & Wijnholds 1996), but under Cu stress is able to release into its surrounding medium Cu-chelators with weak binding strengths (Croot *et al.* 2000), which results in a reduction of metal toxicity.

Glutathione is the main thiol used by eukaryotic cells to uphold reducing conditions for amino acids and proteins, and also to protect the cells against oxidative damage (Fahey *et al.* 1987). Intracellular GSH concentrations are regulated in a dynamic manner which differs between different species and even different strains of the same species. It appears that healthy phytoplankton cells maintain intracellular GSH at a constant level for essential functions, in addition to its use for PC production (Tang *et al.* 2000; Ahner *et al.* 2002). Both depletion (Rijstenbil & Wijnholds 1996; Ahner *et al.* 2002; Morelli & Scarano 2004; this study) and increases (Okamoto *et al.* 2001; Ahner *et al.* 2002; this study) in intracellular GSH concentrations have been reported in various phytoplankton species upon metal exposure. This thiol is therefore not an appropriate indicator of metal stress in phytoplankton.

Tang *et al.* (2000) hypothesised that a high PC_{total}: GSH ratio would be an indication of pronounced metal stress. Comparable PC_{total}: GSH ratios as observed in our study were reported by Rijstenbil & Wijnholds (1996) (see Table 3) for *P. tricornutum* cultures grown in a medium similar to the one employed here and exposed to separate additions of 0.4 μM Cu and 9 μM Cd (24 h incubation period). *P. tricornutum* cultures grown at higher salinity (salinity 35) and subjected to a shorter incubation period (7 h) with individual 10 μM Cd and 10 μM Pb produced the highest PC_{total}: GSH values (Morelli & Scarano 2001). Incubation exposure experiments using lower metal concentrations (pM to nM range) produced PC: GSH ratios around one order of magnitude lower than those for metals in the μM range (Ahner *et al.* 2002). The PC_{Total}: GSH ratios indicated that exposure to Cu caused a stronger stress than exposure to the combination of Cu with Zn or single Cd. Despite the competition between Cd and Zn for metal uptake sites, which should ameriolate Cd toxicity, a higher PC_{total}: GSH value was observed for the exposure to the combination of Cd with Zn than to the single exposure to Cd. According to our experiments using

P. tricornutum, the PC_{Total}: GSH ratios do not provide a unambiguous indication of metal stress as the GSH concentrations in phytoplankton cells are subject to strong dynamic control with important temporal changes in concentration.

The different strains of *P. tricornutum* used by various workers could partly explain the variabilities in the production of GSH and PCs by the different workers. It is worth noting that the *P. tricornutum* employed in the present study has been cultured for more than 90 years in the collection facilities of the Marine Biological Association (Plymouth, UK). Thus, in addition to the natural high metal tolerance of *P. tricornutum* (Torres *et al.* 1997), the biological responses to metal toxicity could be altered through evolution of even more tolerant strains over these years.

Our study indicates that the diatom *P. tricornutum* readily produces PCs upon exposure to enhanced metal concentrations. Other phytoplankton species may use different strategies to alleviate metal stress, e.g. production of extracellular metal chelating ligands. The results from our study showed that PC production in *P. tricornutum* is influenced by combinations of metals. This diatom is a common species in the coastal waters of southwest England. The work presented here therefore shows that PC production by natural phytoplankton assemblages is likely to be a function of the phytoplankton species present, and the occurrence of different toxic metals at enhanced concentrations.

Acknowledgements

SKK thanks Conselho Nacional de Pesquisa Científica e Tecnológica do Brasil (CNPq) for the financial support. The authors acknowledge the European Union Framework 5 IMTEC Project (Contract EVK3-CT-2000-00036) for support. We thank the two anonymous reviewers for their constructive comments.

References

Ahner BA, Kong S, Morel FMM. 1995 Phytochelatin production in marine-algae. 1. An Interspecies Comparison. *Limnol Oceanogr* **40**, 649–657.

Ahner BA, Morel FMM. 1995 Phytochelatin production in marine-algae 2. Induction by various metals. *Limnol Oceanogr* **40**, 658–665.

Ahner BA, Wei LP, Oleson JR, Ogura N. 2002 Glutathione and other low molecular weight thiols in marine phytoplankton under metal stress. *Mar Ecol Prog Ser* **232**, 93–103.

Cid A, Herrero C, Torres E, Abalde J. 1995 Copper toxicity on the marine microalga *Phaeodactylum tricornutum* – effects on photosynthesis and related parameters. *Aquat Toxicol* **31**, 165–174.

Cobbett CS. 2000 Phytochelatins and their roles in heavy metal detoxification. *Plant Physiol* **123**, 825–832.

Croot PL, Moffett JW, Brand LE. 2000 Production of extracellular Cu complexing ligands by eucaryotic phytoplankton in response to Cu stress. *Limnol Oceanogr* **45**, 619–627.

Da Silva JJRF, Williams RJP. 1991 The Biological Chemistry of the Elements. Clarendon, 561 pp..

Faheay RC, Buschbacher RM, Newton GL. 1987 The evolution of glutathione metabolism in phototrophic microorganisms. *J Mol Evol* **25**, 81–88.

Fox TC, Guerinot ML. 1998 Molecular biology of cation transport in plants. *Annu Rev Physiol Plant Mol Biol* **49**, 669–696.

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

Knauer K, Ahner B, Xue HB, Sigg L. 1998 Metal and phytochelatin content in phytoplankton from freshwater lakes with different metal concentrations. *Environ Toxicol Chem* **17**, 2444–2452.

Lee JG, Ahner BA, Morel FMM. 1996 Export of cadmium and phytochelatin by the marine diatom *Thalassiosira weissflogii*. *Environ Sci Technol* **30**, 1814–1821.

Morel FMM, Rueter JG, Anderson DM, Guillard RRL. 1979 Aquil: a chemically defined phytoplankton culture medium for trace metal studies. *J Phycol* **15**, 135–145.

Morelli E, Pratesi E. 1997 Production of phytochelatins in the marine diatom *Phaeodactylum tricornutum* in response to copper and cadmium exposure. *Bull Environ Contam Toxicol* **59**, 657–664.

Morelli E, Scarano G. 1995 Cadmium-induced phytochelatins in marine alga *Phaeodactylum tricornutum* – effect of metal speciation. *Chem Spec Bioavail* **7**, 43–47.

Morelli E, Scarano G. 2001 Synthesis and stability of phytochelatins induced by cadmium and lead in the marine diatom *Phaeodactylum tricornutum*. *Mar Environ Res* **52**, 383–395.

Morelli E, Scarano G. 2004 Copper-induced changes of non-protein thiols and antioxidant enzymes in the marine microalga *Phaeodactylum tricornutum*. *Plant Sci* **167**, 289–296.

Okamoto OK, Pinto E, Latorre LR, Bechara EJH, Colepicolo P. 2001 Antioxidant modulation in response to metal-induced oxidative stress in algal chloroplasts. *Arch Environ Contam Toxicol* **40**, 18–24.

Price NM, Harrison GI, Hering JG, Hudson RJ, Nirel PMV, Palenki B, Morel FMM. 1988 Preparation and chemistry of the artificial algal culture medium Aquil. *Biol Oceanogr* **6**, 443–461.

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

Rijstenbil JW, Wijnholds JA. 1996 HPLC analysis of nonprotein thiols in planktonic diatoms: Pool size, redox state and response to copper and cadmium exposure. *Mar Biol* **127**, 45–54.

Scarano G, Morelli E. 2002 Characterization of cadmium- and lead-phytochelatin complexes formed in a marine microalga in response to metal exposure. *Biometals* **15**, 145–151.

Schecher WD, Mcavoy DC. 1992 Mineql+ – A software environment for chemical-equilibrium modeling. *Comp Environ Urban Syst* **16**, 65–76.

Serkiz SM, Allison JD, Perdue EM, Allen HE, Brown DS. 1996 Correcting errors in the thermodynamic database for the equilibrium speciation model MINTEQA2. *Water Res* **30**, 1930–1933.

Sunda WG, Huntsman SA. 1996 Antagonisms between cadmium and zinc toxicity and manganese limitation in a coastal diatom. *Limnol Oceanogr* **41**, 373–387.

Tang DG, Hung CC, Warnken KW, Santschi PH. 2000 The distribution of biogenic thiols in surface waters of Galveston Bay. *Limnol Oceanogr* **45**, 1289–1297.

Torres E, Cid A, Fidalgo P, Herrero C, Abalde J. 1997 Long-chain class III metallothioneins as a mechanism of cadmium tolerance in the marine diatom *Phaeodactylum tricornutum Bohlin*. *Aquat Toxicol* **39**, 231–246.

Torres E, Cid A, Herrero C, Abalde J. 2000 Effect of cadmium on growth, ATP content, carbon fixation and ultrastructure in the marine diatom *Phaeodactylum tricornutum Bohlin*. *Water Air Soil Pollution* **117**, 1–14.

Twiss MR, Errecalde O, Fortin C, Campbell PGC, Jumarie C, Denizeau F, Berkelaar E, Hale B, van KRees. 2001 Coupling the use of computer chemical speciation models and culture techniques in laboratory investigations of trace metal toxicity. *Chem Spec Bioavailab* **13**, 9–24.

Wei LP, Donat JR, Fones G, Ahner BA. 2003 Interactions between Cd, and Cu, and Zn influence particulate phytochelatin concentrations in marine phytoplankton: laboratory results and preliminary field data. *Environ Sci Technol* **37**, 3609–3618.

Williams LE, Pitman JK, Hall JL. 2000 Emerging mechanisms for heavy metal transport in plants. *Biochim Biophys Acta* **1465**, 104–126.