

# Glutathione and other low molecular weight thiols in marine phytoplankton under metal stress

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**ABSTRACT:** Low molecular weight intracellular sulfhydryl-containing compounds are responsible for the intracellular detoxification of metals such as Cd and Cu in eucaryotic phytoplankton. Glutathione, the most abundant thiol in non-stressed cells (0.8 to 2.8 mM), chelates metals directly, and also serves as the biochemical precursor from which phytochelatins are synthesized. Very little is known about physiological variations in glutathione concentrations under conditions of metal stress or other environmental factors. We found that in response to steady-state Cd and Cu exposure, glutathione concentrations remain remarkably constant in all of the algae examined, even as phytochelatin concentrations increase by up to 100-fold control values (in some cases exceeding glutathione). In some species,  $\gamma$ -glu-cys and cysteine, precursors to glutathione, also increase significantly in response to metal exposure. Short-term exposure to Cd and Cu can have a dramatic effect on the intracellular glutathione and phytochelatin concentration especially when the culture is under nutrient limitation.

**KEY WORDS:** Glutathione · Phytochelatin · Thiols · Detoxification · Marine phytoplankton

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## INTRODUCTION

Several recent papers have suggested that glutathione may be an important ligand for some metals in surface seawater (Le Gall & van den Berg 1998, Leal et al. 1999, Tang et al. 2000) but relatively little is known about intracellular concentrations of glutathione or fluctuations thereof in marine phytoplankton. In the laboratory, concentrations are generally similar to those measured in higher plants and other organisms (Rijstenbil & Wijnholds 1996) but environmental factors such as light and nutrient availability may be important in the field (Matrai & Vetter 1988, Rijstenbil et al. 1998). Metal concentrations are also likely to play a role, since phytochelatins, metal-detoxification peptides, are produced from glutathione.

Glutathione ( $\gamma$ -glutamylcysteinylglycine) is found in all eucaryotes and it is the most abundant small peptide and the dominant cellular thiol in many living

systems. It is typically present at millimolar concentrations within cells and can constitute up to 90% of non-protein thiols (Giovanelli et al. 1980). The gamma linkage within glutathione confers an unusually high resistance to intracellular peptidases and thus it can be maintained at relatively high concentrations (Giovanelli et al. 1980). Glutathione is synthesized by the action of 2 enzymes:  $\gamma$ -glutamylcysteine synthetase and glutathione synthetase, which have been found to occur in the cytosol and chloroplasts of eucaryotic plant cells (Bergmann & Rennenberg 1993).

Glutathione is a multifunctional tri-peptide and although not fully understood, its primary intracellular functions are as summarized in Fig. 1. One of its major roles is as a line of defense against reactive oxygen derivatives because of the ease with which glutathione undergoes oxidation and reduction reactions. The sulfhydryl group on the cysteine residue often functions as a site of conjugation, such as in the detoxification of organic compounds (e.g. herbicides) through the action of a family of enzymes called glutathione-S-transferases (Marrs 1996) and in its putative role as a

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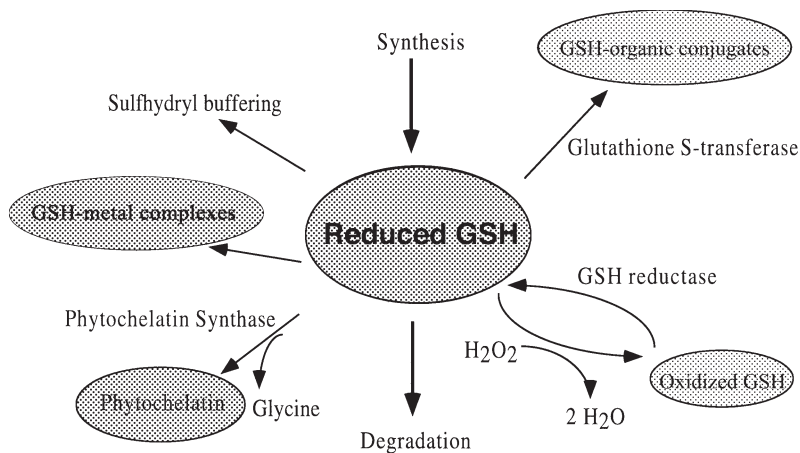


Fig. 1. Proposed functions and pools of glutathione (GSH) in a eucaryotic algal cell

carrier in the sulfate assimilation pathway (Giovanelli et al. 1980). In addition, glutathione is also the primary precursor from which phytochelatins are made. Phytochelatins are small, sulfhydryl-containing peptides with the formula  $(\gamma\text{-glutamylcysteinyl})_n\text{-glycine}$ , where  $n$  varies from 2 to 11 (Grill et al. 1985), although the  $n = 2, 3$  and 4 oligomers are predominant in phytoplankton (Ahner et al. 1995). They are synthesized rapidly in response to some heavy metals (such as Cd and Cu), even at very low concentrations, by the constitutive enzyme phytochelatin synthase that catalyzes the transfer of  $\gamma\text{-glu-cys}$  from glutathione to other glutathione molecules to form phytochelatin  $n = 2$  or to  $(\gamma\text{-glu-cys})_n\text{-gly}$  polymers to form phytochelatin chains of length  $n + 1$  (Grill et al. 1989). Recent work by Vatamaniuk et al. (2000) using the purified recombinant phytochelatin synthase from *Arabidopsis thaliana* has shown that both the Cd-bis-glutathione complex and free glutathione serve as substrates for the enzyme, which explains the metal-dependent reaction mechanism. An additional synthesis mechanism, in which free  $\gamma\text{-glu-cys}$  units are polymerized and then a glycine residue is added by glutathione synthetase, has been identified in fission yeast (Hayashi et al. 1991). Glutathione availability in plant cells has been shown to limit metal tolerance and phytochelatin synthesis (Zhu et al. 1999).

Both glutathione and phytochelatin chelate heavy metals such as Cd, Cu, and Pb via sulfhydryl coordination (Rabenstein 1989; Strasdeit et al. 1991). While the binding constants for the various phytochelatin oligomers are not known, the longer peptides form stronger complexes with metals (Mehra et al. 1995). Given the abundance of glutathione in algal cells, it may be an important chelator of some metals. While studies of metal-stressed plants and algae have reported significant fluctuations in glutathione concen-

tration upon the onset of phytochelatin synthesis (both increases and decreases have been observed: Ruegsegger et al. 1990, Rauser et al. 1991, Rijstenbil & Wijnholds 1996), there have been few studies that have examined the effect of steady state exposure to metals on glutathione concentrations.

Ultimately a major source of glutathione in surface seawater is the eucaryotic phytoplankton. Here we examined the intracellular concentrations of glutathione and other low molecular weight thiols in several species of eucaryotic marine algae under steady state exposure to a range of Cd and Cu concentrations, as well as during short-term additions of these same

metals. We also investigated whether other factors, such as light levels and nutrient status, influence glutathione and phytochelatin concentrations.

## MATERIALS AND METHODS

**Algal culture preparation.** Several species of eucaryotic unicellular marine algae were cultured axenically at  $120 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  and  $19 \pm 1^\circ\text{C}$  in Aquil, a defined medium developed specifically for studies of trace metal nutrition and toxicity in marine algae (Price et al. 1988/89). Species included the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (both from Algensammlung [Collection of Algae] at Göttingen University, Germany), and *T. weissflogii* (CCMP 1336; Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine, USA). *P. tricornutum* is a pennate diatom, while *T. pseudonana* and *T. weissflogii* are centric diatoms. *Emiliania huxleyi* (CCMP 373) is a coccolithophore, or prymnesiophyte. *Dunaliella* sp. (CCMP 367) is a green alga of the order Volvocales. Approximate sizes and chlorophyll contents of the various species are given in Table 1. Culture medium and vessels were prepared to minimize metal contamination.

Aquil employs an EDTA buffer to fix trace metal concentrations in solution. Cd and Cu concentrations were varied in the medium by additions from stock solutions of equimolar Cd or Cu and EDTA (Table 2). When metals are buffered by EDTA, it ensures an approximate steady state supply of free metal ions in the medium as metals are removed by the algae. Equilibrium calculations were performed with MINEQL (Westall et al. 1976) to determine steady state concentrations of free metal ions ( $[\text{Me}^{2+}]$ , reported as  $\text{pMe} = -\log [\text{Me}^{2+}]$ ). For

Table 1. Cellular volume and chl *a* contents of algal species used in calculations of phytochelatin concentrations

Phytoplankton species	Shape	Volume <sup>a</sup> ( $\mu\text{m}^3$ )	Chl <i>a</i> (pg cell <sup>-1</sup> )
<i>Dunaliella</i> sp.	Ovate	210	0.48
<i>Emiliania huxleyi</i> (BT6)	Spherical	13	0.075
<i>Phaeodactylum tricorutum</i>	Elliptical	160	0.23
<i>Thalassiosira pseudonana</i> (3H)	Cylindrical	73	0.26
<i>Thalassiosira weissflogii</i> (Actin)	Cylindrical	1230	4.0

<sup>a</sup>Calculated from average microscopic measurements of cell dimensions

most metal-stressed cultures, the total trace metal concentrations were reduced 10-fold (resulting in 10  $\mu\text{M}$  EDTA as indicated in Table 2) in order to reduce the total EDTA concentration. The inorganic concentrations of all other metals were fixed at Aquil concentrations (Price et al. 1988/89). N, P, and Si concentrations were 10, 300 and 100  $\mu\text{M}$  respectively.

To measure the variation of cellular glutathione and other low molecular weight thiols in response to steady state exposure to Cd and Cu, algae were pre-cultured for at least 2 generations in 30 ml polycarbonate tubes in medium containing added metals at the desired concentrations before transfer to 500 ml acid-cleaned polycarbonate bottles containing the same experimental medium. Algae growth was monitored by measuring *in vivo* fluorescence (Turner 10-AU Fluorometer). Cell densities were determined microscopically with a Levy Hemacytometer (Hausser Scientific Company) and with a Coulter Counter (Coulter Electronics). Near the end of exponential growth, cells were collected by gentle filtration (<5 psi) onto 25 mm glass-fiber filters (Whatman GF/F or GF/A) and were frozen in liquid nitrogen. Frozen samples of *Dunaliella* sp. and *Phaeo-*

Table 2. Concentrations ( $\mu\text{M}$ ) of equimolar metal-EDTA added to achieve the listed free metal concentrations in Aquil. pMe is equivalent to  $-\log [\text{Me}^{2+}]$ . TM EDTA indicates the concentration of EDTA present in the medium prior to adding Cd or Cu. Compared to standard Aquil medium, 10-fold less trace metal mix is used for the 2 highest Cd treatments and all the Cu treatments

	pMe = 12	pMe = 11	pMe = 10	pMe = 9
<b>Cd</b>				
Cd-EDTA	0.03	0.3	0.3	3
TM EDTA	100	100	10	10
<b>Cu</b>				
Cu-EDTA	0.12	1.2	12	120
TM EDTA	10	10	10	10

*dactylum tricorutum* showed no significant loss of either glutathione or  $\gamma$ -glu-cys for up to 6 wk of storage in liquid nitrogen (data not shown).

To study temporal variations of intracellular phytochelatin and glutathione in response to short-term metal exposure (pMe = 9), Cd-EDTA or Cu-EDTA was added to exponential and stationary (5 to 6 d after the end of exponential growth) cultures grown in control medium. Cd incubations were performed with cultures of *Thalassiosira pseudonana*, *Emiliania huxleyi*, and *Dunaliella* sp. and 1 Cu

experiment was performed with *E. huxleyi*. Samples were collected by gentle filtration from  $t = 0$  to 36 h for thiol and chl *a* measurements. Samples from parallel control cultures were also collected.

Chl *a* was measured by extraction in aqueous acetone (Parsons et al. 1984). Filtered cells were placed into 10 ml of cold 90 % acetone (10 % water) and stored overnight at 4°C in the dark. Fluorescence was measured with a Turner fluorometer and the concentration of chl *a* was calculated using a calibration curve developed from a spectrophotometrically calibrated solution of chl *a* extracted from spinach.

**Sample preparation for HPLC analysis.** The samples were removed from storage in liquid nitrogen and immediately plunged into 2.5 ml of 10 mM methanesulfonic acid (MSA) at 70°C for 2 min to denature the proteins, before they were homogenized for 2 to 3 min using a Wheaton Overhead Stirrer in an ice-water bath. The resulting slurry was then centrifuged for 10 min at 13000 rpm at 4°C (Biofuge Fresco, Heraeus Instruments), and the supernatant was retained and diluted, if necessary, with 10 mM MSA for subsequent derivatization with monobromobimane (mBBr) (Molecular Probes) generally following the protocol described in Ahner et al. (1995), which was originally based on the method of Newton et al. (1981). Changes from the method published by Ahner et al. (1995) included 10-fold lower probe and reductant concentrations as well as the addition of excess dithiothreitol after the reaction incubation to utilize excess unreacted probe.

**HPLC method.** Samples were analyzed by reverse-phase HPLC on a C<sub>8</sub> column (2.1 × 250 mm, Alltech Solvent Miser). The compounds of interest were quantified by post-column fluorescence detection (Gilson, excitation 310 to 410 nm and emission 475 to 650 nm) of the mBBr probe. The flow rate was constant at 0.2 ml min<sup>-1</sup>. A gradient of acetate buffer (Solution A: 0.25 % acetic acid buffered to pH = 4 with NaOH; 8 % acetonitrile; 0.1 mM tetrahexylammonium bromide, Aldrich Chemical) and 100 % acetonitrile (Solution B) was used to separate thiols. The elution gradient used was as

follows: 0% B for 10 min, a linear increase to 25% B over 5 min, and another more gradual linear increase to 50% B over 50 min. This was followed by an additional 30 min of 100% A to re-equilibrate the column. Glutathione, cysteine and  $\gamma$ -glu-cys (Sigma) and phytochelatin standards ( $n = 2, 3$  and  $4$ ; synthesized by the New York Center for Advanced Technology, Biotechnology Program) were used to identify thiol peaks and to develop calibration curves for the conversion of peak area to concentration. Using a 200  $\mu$ l injection loop resulted in a detection limit of  $\sim 1$  pmol of the  $n = 2$  oligomer.

## RESULTS

### Glutathione

Five species of marine algae were exposed to variable concentrations of Cd and Cu and intracellular glutathione concentrations were measured. Control cultures (without metal addition) contained remarkably similar amounts of glutathione—from 475 to 725  $\mu$ mol g chl  $a^{-1}$  (Fig. 2). Although *Dunaliella* sp. appeared to have slightly higher concentrations than the other algae, an ANOVA analysis comparing control concentrations revealed no significant differences among algae ( $p > 0.05$ ). When concentrations were converted to  $\mu$ moles per cell volume, glutathione concentrations ranged from 0.78 to 2.4 mM (780 to 2400  $\mu$ M; Table 3). These values agree well with concentrations reported in other studies; for example, Rijstentbil & Wijnholds (1996) reported values ranging from 0.1 to 3.3 mM (100 to 3300  $\mu$ M) in 4 marine algae.

As Cd and Cu increased in the medium, there was relatively little change in glutathione concentrations and no truly discernable pattern of change among species (Fig. 2). Two-way ANOVA indicated no significant effect of Cd or Cu on glutathione ( $p > 0.05$ ). However, for individual species there may be a dose-response. Glutathione in *Emiliania huxleyi* seemed to decrease at high Cu concentrations, while in *Phaeodactylum tricornerutum* and *Dunaliella* sp. it showed an increase at pCu = 9 and pCd = 9, respectively.

### Cysteine and $\gamma$ -glu-cys

Concentrations of cysteine and  $\gamma$ -glu-cys in control cultures were significantly less than that of gluta-

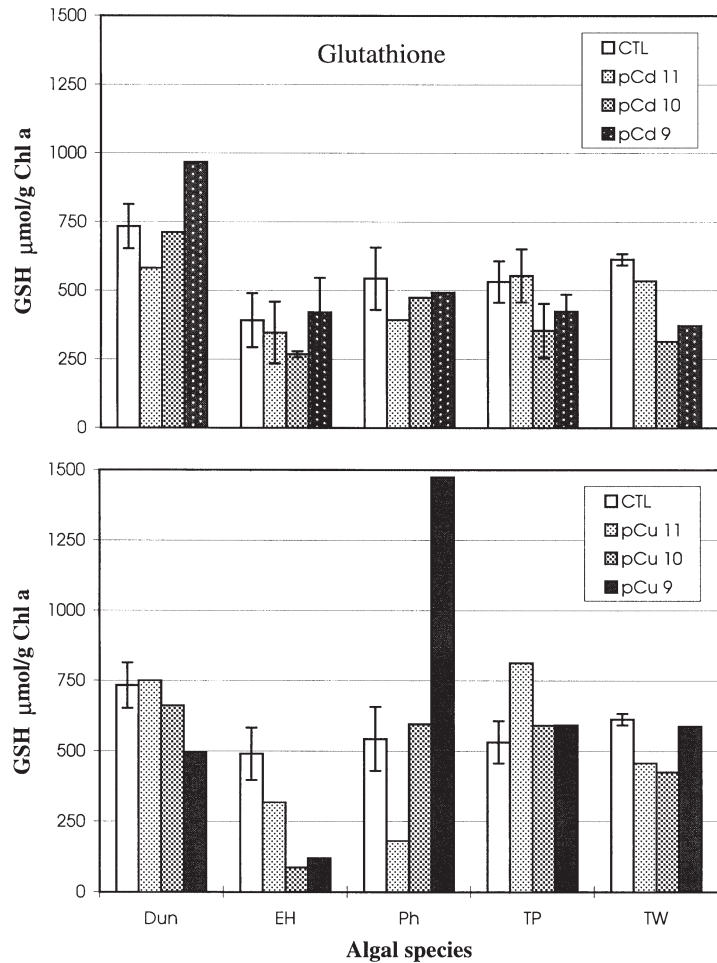


Fig. 2. Glutathione concentrations (GSH,  $\mu$ mol g chl  $a^{-1}$ ) in marine algae at varying concentrations of cadmium (top) and copper (bottom). Dun: *Dunaliella* sp.; EH: *Emiliania huxleyi*; Ph: *Phaeodactylum tricornerutum*; TP: *Thalassiosira pseudonana*; TW: *T. weissflogii*. Error bars represent range of 2 (duplicate) cultures; data points without error bars were not duplicated

thione, and much more variability was observed among species (Fig. 3). In most of the algal species examined these compounds were 100-fold less than glutathione, whereas in *Emiliania huxleyi* cysteine concentrations were half and  $\gamma$ -glu-cys was about one-third that of glutathione. Measured concentrations of cysteine and  $\gamma$ -glu-cys ranged from below detection to  $\sim 200$   $\mu$ mol g chl  $a^{-1}$ .

In response to Cd exposure, both cysteine and  $\gamma$ -glu-cys increased with increasing amounts of Cd in *Emiliania huxleyi*, whereas only  $\gamma$ -glu-cys increased in *Thalassiosira weissflogii* and *Phaeodactylum tricornerutum*. Concentrations remained constant in *T. pseudonana* and remained below detection in *Dunaliella* sp. In *E. huxleyi*, both  $\gamma$ -glu-cys and cysteine exceeded glutathione concentrations at the highest Cd concentra-

Table 3. Glutathione (GSH), phytochelatin and metal concentrations ( $\mu\text{M}$ ) in each alga. Values are  $\mu\text{mol}$  per cell volume; conversions from values in Figs. 2 to 4 based on cellular volume of control cells in Table 2. Total phytochelatin (PC) is the sum of the  $\gamma\text{-glu-cys}$  units in the individual oligomers ( $\sum \gamma\text{-glu-cys} = 2[n = 2] + 3[n = 3] + 4[n = 4]$ ) where  $n$  = number of repeating units. bd: below detection

Species	GSH	n = 2	n = 3	n = 4	Total PC	Metal
<i>Dunaliella</i> sp.						
Control	1700	2.7	1.2	0.23	10	
pCd = 12 <sup>a</sup>	1700	2.3	2.7	1.5	19	
pCd = 11	1300	14	5.3	5.4	66	
pCd = 10	1600	47	29	52	390	
pCd = 9	2200	61	65	56	540	
pCu = 12	1500	bd	bd	bd	bd	
pCu = 11	1700	bd	bd	bd	bd	
pCu = 10	1500	33	14	2.9	120	
pCu = 9	1100	30	6.9	0.78	84	
<i>Emiliania huxleyi</i>						
Control	2400	bd	bd	bd	bd	
pCd = 11	2000	320	180	270	2300	460 <sup>b</sup>
pCd = 10	1500	410	490	560	4500	1000 <sup>b</sup>
pCd = 9	2400	430	470	240	3200	640 <sup>b</sup>
pCu = 11	1800	bd	bd	bd	bd	280 <sup>b</sup>
pCu = 10	500	290	310	230	2400	2100 (10.5) <sup>c</sup>
pCu = 9	690	46	58	17	330	
<i>Phaeodactylum tricornutum</i>						
Control	780	6	4.2	4.4	42	
pCd = 11	560	2.2	bd	0.69	7.1	
pCd = 10	680	43	4.0	6.4	120	
pCd = 9	710	40	3.6	8.0	120	
pCu = 11	260	41	32	8.1	210	
pCu = 10	860	88	22	6.3	270	
pCu = 9	2100	160	87	bd	580	
<i>Thalassiosira pseudonana</i>						
Control	1900	44	5.3	2.2	110	
pCd = 12	2100	53	14	bd	147	0.56 <sup>d</sup>
pCd = 11	2000	820	230	7	2300	7 <sup>d</sup>
pCd = 10	1300	1700	1300	200	8100	70 <sup>d</sup>
pCd = 9	1500	1500	1800	330	9700	
pCu = 11	2900	110	bd	bd	220	250 <sup>c</sup>
pCu = 10	2100	400	240	29	1600	
pCu = 9	2100	350	270	27	1600	
<i>Thalassiosira weissflogii</i>						
Control	2000	bd	bd	bd	bd	
pCd = 12	1800	44	36	15	250	15 <sup>b</sup>
pCd = 11	1700	200	25	7.7	500	16 <sup>b</sup>
pCd = 10	1000	970	450	160	3900	25 <sup>b</sup>
pCd = 9	1200	1200	690	140	5000	64 <sup>b</sup>
pCu = 12	1900	bd	bd	bd	bd	4.6 <sup>c</sup>
pCu = 11	1500	15	13	5.9	93	50 <sup>c</sup>
pCu = 10	1400	28	12	5.9	120	177 (10.5) <sup>c</sup>
pCu = 9	1900	15	15	6.8	100	

<sup>a</sup>pCd =  $-\log[\text{Cd}^{+2}]$  in growth medium  
<sup>b</sup>Ahner et al. (1995)  
<sup>c</sup>Sunda & Huntsman (1995), in parentheses: metal concentration of medium if different from table  
<sup>d</sup>Sunda & Huntsman (1998)

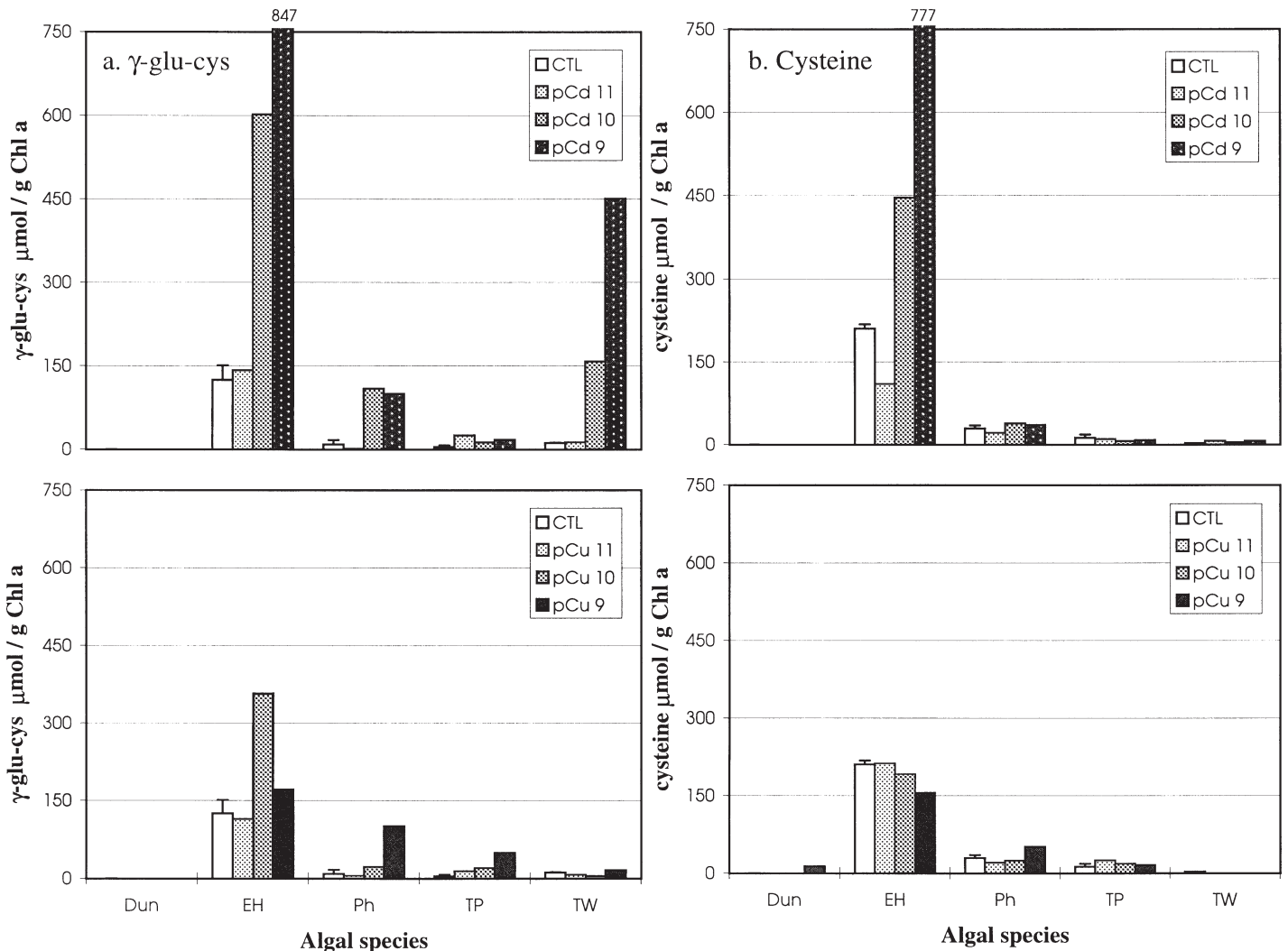


Fig. 3. Concentrations ( $\mu\text{mol g chl}^{-1}$ ) of  $\gamma$ -glu-cys (a), and cysteine (b) in marine algae at varying concentrations of cadmium (top) and copper (bottom). The off scale data points in (a) and (b) are as indicated on the graph. Abbreviations and error bars are as in Fig. 2. The absence of a bar indicates that the compound was below detection. Note difference in y-axis scale for these graphs compared to Figs. 2 & 4

tion. Cysteine concentrations in a later experiment with a culture of *E. huxleyi* grown in a light:dark cycle (16:8) were 1056, 2262, 1439 and 1932  $\mu\text{mol g chl}^{-1}$  with increasing Cd. These higher values may be the result of a diurnal cycle in cysteine concentrations (cultures in Fig. 3b were grown under constant light). Rates of sulfate reduction and assimilation follow a diurnal cycle in higher plants (Kopriva et al. 1999).

In response to Cu, again the greatest response was seen in *Emiliania huxleyi* for  $\gamma$ -glu-cys (*Thalassiosira pseudonana* and *Phaeodactylum tricornerutum* increased slightly at the highest Cu concentration), but concentrations of cysteine remained constant for all algae at all Cu concentrations. The more frequently observed increase in  $\gamma$ -glu-cys is consistent with other

published experiments in which  $\gamma$ -glu-cys synthetase activity was more stimulated than the activities of other enzymes in the biosynthetic pathway of glutathione in Cd-exposed tissues (Schäfer et al. 1998).

### Phytochelatin

Phytochelatin were measured in control cultures of *Dunaliella* sp., *Thalassiosira pseudonana* and *Phaeodactylum tricornerutum*, but were below detection in the other 2 species (Table 3). Our inability to measure phytochelatin in the control cultures of *T. weissflogii* and *Emiliania huxleyi* was likely due to insufficient biomass in those particular samples, since, in an earlier

study employing the same growth medium, these organisms were found to contain 13 and 40  $\mu\text{mol } \gamma\text{-glu-cys gchl}^{-1}$  as phytochelatin respectively (Ahner et al. 1995).

Upon exposure to Cd or Cu there was a significant increase in the intracellular concentration of phytochelatin in each of the species studied (Fig. 4), confirming the results of Ahner et al. (1995). In general, absolute quantities of phytochelatin synthesized in response to Cu are lower than in Cd-stressed cells. The one exception to this trend was *Phaeodactylum tricornutum*, which produced the least amount of phytochelatin in response to Cd and was among those producing the most in response to Cu. *Dunaliella* sp. and *Thalassiosira weissflogii* synthesized very little phytochelatin in response to Cu; it is possible that these organisms utilize some alternate detoxification strategy for Cu. Decreases observed in phytochelatin at the highest Cd and Cu concentrations in *Emiliana huxleyi* and a slight decrease observed by Ahner & Morel (1995) at the highest Cu concentration in *T. weissflogii* is also evidence that direct intracellular chelation by phytochelatin alone is not the only detoxification mechanism at work.

### Time-course experiments

Short-term metal addition experiments were performed to examine how glutathione and other thiol constituents varied as function of time after exposure to Cd and Cu. These experiments were performed on exponentially growing cells and on cultures that had been in a stationary phase for 5 to 6 d; this allowed us to compare cells that were presumably nutrient-sufficient to those that were limited by 1 or more nutrients. Cd addition experiments were performed on *Thalassiosira pseudonana*, *Emiliana huxleyi*, and *Dunaliella* sp. and a Cu addition experiment was performed on *E. huxleyi*.

Senescent and exponential cultures of *Thalassiosira pseudonana* responded very similarly upon exposure to Cd with respect to glutathione and phytochelatin production (Fig. 5). Starting concentrations of glutathione were roughly the same in both cultures; they gradually decreased over the first 10 h and then leveled off. Phytochelatin increased in both cultures at the same rate and the final concentrations approached the steady state concentration measured at pCd = 9. The final phytochelatin concentration in the exponential

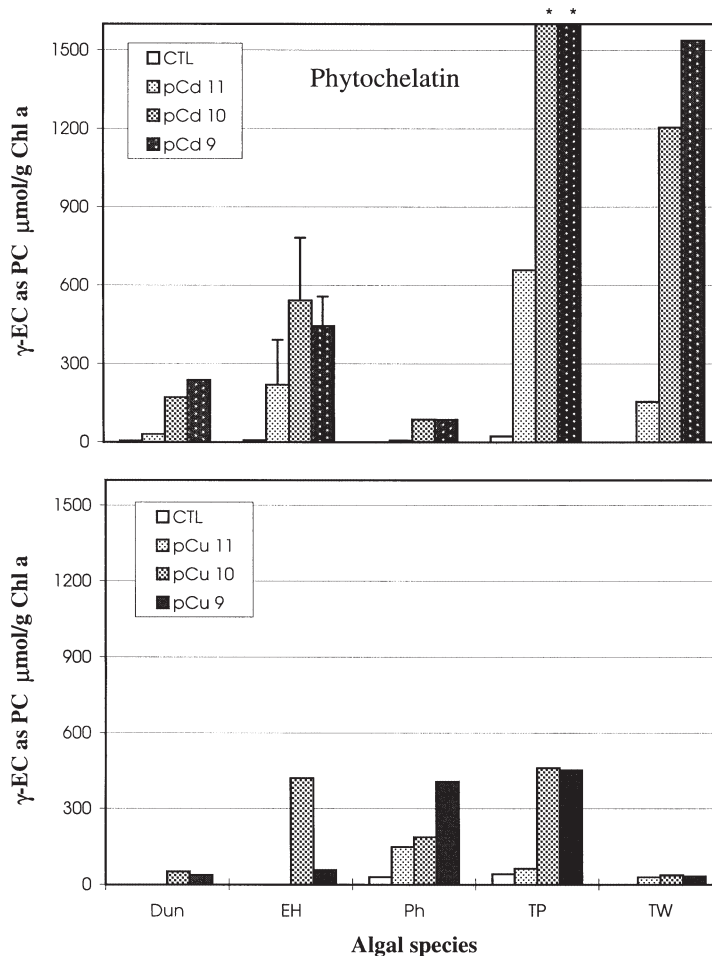


Fig. 4. Concentrations ( $\mu\text{mol g chl}^{-1}$ ) of phytochelatin, in units of  $\gamma$ -glu-cys ( $\Sigma \gamma\text{-EC} = 2[n = 2] + 3[n = 3] + 4[n = 4]$ ) in marine algae at varying concentrations of cadmium (top) and copper (bottom). Abbreviations and error bars are as described in Fig. 2. \*: off-scale values of total  $\gamma\text{-EC}$  are 2300 and 2700  $\mu\text{mol g chl}^{-1}$  for pCd 10 and 9 respectively

cells was slightly higher than in the senescent cells and the reverse was true for the glutathione. In control cultures (no added Cd) glutathione remained constant and phytochelatin was below detection for the duration of the experiment (data not shown).

As with *Thalassiosira pseudonana*, in both exponential and senescent Cd-treated cultures of *Dunaliella* sp., glutathione was maintained at very similar levels after 10 and 30 h of exposure. However, after this same time interval, phytochelatin was 10 to 20 times higher in the exponential culture than in the senescent culture (data not shown).

For *Emiliana huxleyi*, on the other hand, after the first few hours of exposure to Cd both glutathione and phytochelatin were significantly higher in the exponential culture than in the senescent culture (Fig. 6). In both cultures the glutathione declined initially, but in

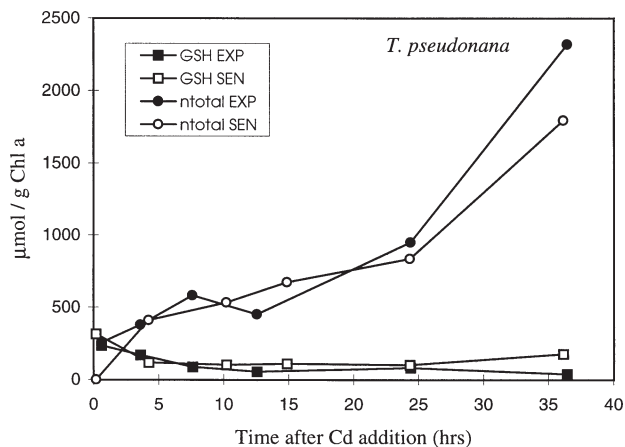


Fig. 5. *Thalassiosira pseudonana*. Time course of glutathione (GSH) and phytochelatin ( $ntotal = \sum \gamma\text{-EC} = 2[n = 2] + 3[n = 3] + 4[n = 4]$ ) concentrations upon exposure to  $pCd = 9$  at  $t = 0$ . Filled symbols represent concentrations in exponentially growing cells and open symbols concentrations in senescent-phase cells. Upon Cd addition, chl *a* increased similarly in both treated and control exponential cultures, whereas it remained constant in both the treated and control senescent cultures (data not shown)

the exponential culture glutathione recovered dramatically (peaking at 5 h) and then maintained a constant though elevated concentration from 10 to 36 h; in the senescent culture, glutathione continued to decrease and then leveled off after 10 h. Phytochelatin peaked early in the senescent culture ( $t = 1$  h) and then tapered off, while in the exponential culture it peaked at 5 h and then gradually climbed back to near 700  $\mu\text{mol gchl a}^{-1}$ . Final concentrations of both glutathione and phytochelatin were similar to those measured in steady state cultures at the same Cd concentration.

Similar to what we observed with Cd addition, exponentially growing *Emiliana huxleyi* synthesized excess glutathione in response to Cu, while the senescent cells steadily lost glutathione (Fig. 7). Phytochelatin was rapidly produced by the exponential cells over the first 5 h and then leveled off at near steady state values ( $\sim 100 \mu\text{mol gchl a}^{-1}$ ) (data not shown). In senescent cells, phytochelatin was below detection for the entire experiment.

In the Cd experiment, it is unclear whether the peak at 5 h in Fig. 6 was real or an artifact, since these experiments were not duplicated. However, a similar peak also appeared for  $\gamma$ -glu-cys in the Cd experiment as well as for  $\gamma$ -glu-cys, cysteine, and phytochelatin in the Cu addition experiment (data not shown).

Sensitivity to metals was also different among the species examined. Growth rates were unaltered by the addition of Cd to the exponential cultures of both *Thalassiosira pseudonana* and *Dunaliella* sp. and the chl *a* concentration remained constant in both the treated

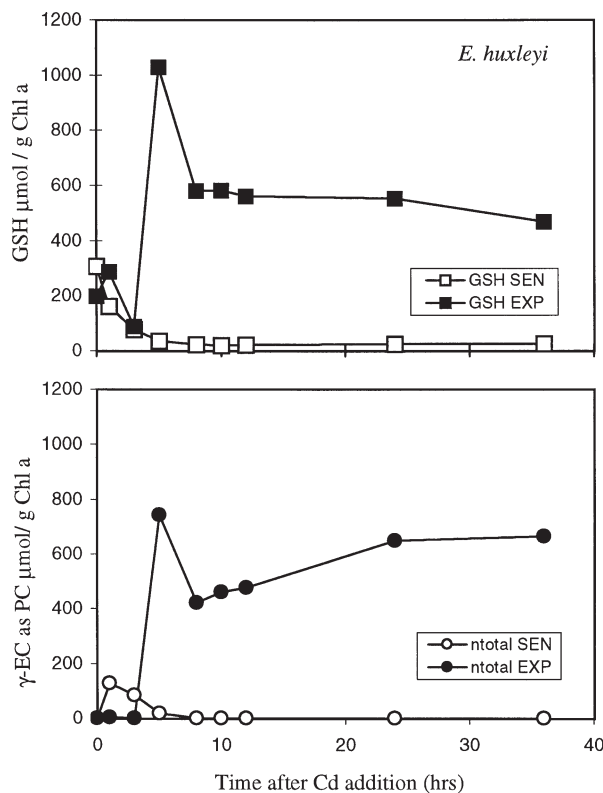


Fig. 6. *Emiliana huxleyi*. Time course of glutathione (GSH; top) and phytochelatin ( $ntotal = \sum \gamma\text{-EC} = 2[n = 2] + 3[n = 3] + 4[n = 4]$ ; bottom) concentrations upon exposure to  $pCd = 9$  at  $t = 0$ . Filled symbols represent concentrations in exponentially growing cells and open symbols concentrations in senescent-phase cells. Upon Cd addition, the growth rate of exponential cells (as determined by chl *a*) decreased by 35% compared to control. Total chl *a* decreased steadily in both treated and control senescent cells (data not shown)

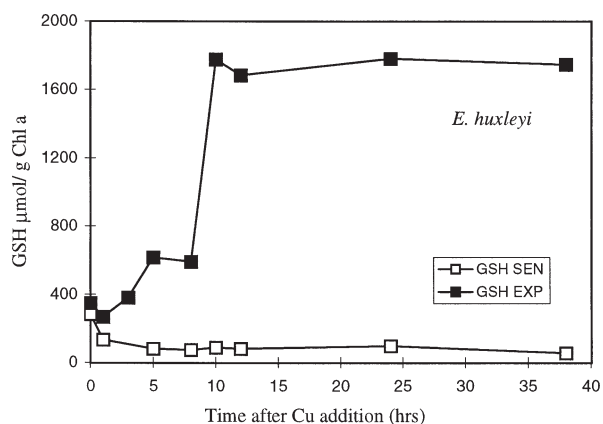


Fig. 7. *Emiliana huxleyi*. Time course of glutathione (GSH) concentrations upon exposure to  $pCu = 9$  at  $t = 0$ . ■: concentrations in exponentially growing cells; □: concentrations in senescent-phase cells. Upon Cu addition, chl *a* initially declined in treated exponential cells and then recovered after 5 h, resuming growth at a rate about one-third that of controls (data not shown). Chl *a* in treated and control senescent cultures decreased similarly (data not shown)

and control senescent cultures. *Emiliana huxleyi*, on the other hand, appeared to be sensitive to Cd and Cu additions. Relative to controls, growth rates decreased in both Cd- and Cu-treated exponential cultures (details in Fig. 6 and 7 legends). A decrease in chl *a* was observed in both treated and control senescent cultures. It is possible that the sensitivity of *E. huxleyi* to the added metals contributed to the observed differences in glutathione and phytochelatin.

## DISCUSSION

Intracellular glutathione concentrations are quite similar from species to species and in general are not significantly altered by long-term exposure to Cd and Cu. Glutathione appears to be tightly regulated, despite substantial utilization for phytochelatin synthesis, which is consistent with the cell's need to maintain glutathione for other essential functions. Conversely,  $\gamma$ -glu-cys and cysteine (in 1 case), the main precursors of glutathione are significantly elevated in some algae in response to metal exposure. As in earlier studies, greater phytochelatin production is generally observed in response to Cd rather than Cu, although *Phaeodactylum tricornutum* is a noted exception in this study.

Perturbations in glutathione concentrations are observed during metal addition experiments and under conditions of nutrient limitation. We observed that some species are unable to synthesize phytochelatin or maintain high levels of glutathione once the culture has reached senescence. One clear limitation of using senescent cultures in these experiments is that we have not imposed a particular nutrient limitation. Of the 3 algae examined, senescent *Thalassiosira pseudonana* cells appear uniquely able to maintain glutathione and also produce copious amounts of phytochelatin upon addition of cadmium. As silica frustule-forming diatoms, they are ultimately limited by silicate in our culture medium whereas the other species examined are more likely to be limited by nitrogen or phosphorus (N. Price pers. comm.). Nitrogen limitation has been found to limit sulfate assimilation (Koprivova et al. 2000) and thus may limit glutathione synthesis (Rijstenbil et al. 1998), although nitrogen in glutathione represents a very small fraction of the total cellular nitrogen (<0.5% in *T. weissflogii*). In follow-up experiments, batch cultures of nitrogen-limited *T. pseudonana* did not exhibit lower glutathione or phytochelatin production upon Cd addition (data not shown). Phosphorus limitation, on the other hand, may cause ATP deficiencies that might disrupt membrane transport processes in addition to limiting sulfate reduction, the first step of which involves ATP (Giovanelli et al. 1980).

Although glutathione remains constant, phytochelatin concentrations are highly variable among species, especially at high Cu and Cd concentrations. A comparison of Cu-challenged *Thalassiosira weissflogii* and *Emiliana huxleyi* reveals fairly large differences in phytochelatin concentrations that are also observed in cellular Cu concentrations (Sunda & Huntsman 1995; data reported in Table 3). Remarkably, phytochelatin and Cu concentrations are very similar. This is not entirely surprising since phytochelatin production is dependent on the presence of metals in the cell. Not all differences in phytochelatin production, however, can be explained by differences in metal quotas. Other detoxification mechanisms will also affect total phytochelatin concentrations. Cd has been shown to accumulate in vacuoles as a complex with phytochelatin (Salt & Wagner 1993, Ortiz et al. 1995), as ionic Cd (Salt & Rauser 1995) and as a glutathione complex (Li et al. 1997) in yeast and higher plants. In addition, the more stable sulfide-containing phytochelatin-metal clusters (Reese & Winge 1988) may be produced by some algae, which would result in less total phytochelatin. Also, other intracellular chelators may be synthesized; metallothionein genes have been found in several eucaryotic plants (e.g. Zhou & Goldsbrough 1995) and metallothioneins have a greater affinity for both Cd and Cu than the phytochelatin (Weber et al. 1988).

One observation that is difficult to explain with any of the above mechanisms is the large excess of phytochelatin produced in some algae in response to Cd (Table 3). At pCd 11 and 10, phytochelatin concentrations in *Thalassiosira pseudonana* are 2300 and 8100  $\mu\text{M}$  respectively whereas Cd concentrations, as measured by Sunda & Huntsman (1998), are 7 and 70  $\mu\text{M}$  ( $\mu\text{mol l}^{-1}$  cell volume). Assuming an optimal 4:1 stoichiometry of cysteinyl-S:Cd (Strasdeit et al. 1991), these phytochelatin concentrations are 80 and 30 times greater than needed for direct complexation. Similar ratios were observed in *T. weissflogii* (Table 3 and Ahner et al. 1995) but not in the related species *T. oceanica* (Ahner et al. 1995). One possible explanation for this observation is that the phytochelatin synthase in these 2 closely related organisms (*T. weissflogii* and *T. pseudonana*) does not require the Cd-(glutathione)<sub>2</sub> complex as a substrate (as does the *Arabidopsis* form: Vatamaniuk et al. 2000) and the production of the enzyme is under transcriptional control. It is also possible that phytochelatin synthesis in these organisms proceeds via  $\gamma$ -glu-cys polymerization, as has been observed in fission yeast (Hayashi et al. 1991). This could explain the high ratio of phytochelatin to Cd observed in *T. weissflogii* (the forward reaction may be driven by excess substrate), but does not explain the *T. pseudonana* data, nor does excess  $\gamma$ -glu-cys in *Emiliana huxleyi* result in high Cd to phytochelatin ratios.

Further studies on the properties of phytochelatin synthase in marine algae are now possible, since several genes for phytochelatin synthase have been identified (Cobbett 2000). Utilizing conserved regions present in all of the sequenced enzymes, we may be able to obtain the sequence for the synthase in *Thalassiosira weissflogii* and characterize its apparently unique activity.

Particulate concentrations of glutathione in the field are lower than those measured in this study. Matrai & Vetter (1988) reported concentrations of 40 to 100  $\mu\text{mol g chl a}^{-1}$  in surface waters of Saanich Inlet and ~100 to 200  $\mu\text{mol g chl a}^{-1}$  in surface waters of the Southern California Bight (calculated from values plotted in graphs). Tang et al. (2000) measured very low glutathione concentrations in Galveston Bay, Texas, ~10  $\mu\text{mol g chl a}^{-1}$ , with concentrations increasing up to ~170  $\mu\text{mol g chl a}^{-1}$  at the mouth of the bay. In addition, we found that glutathione concentrations in the Elizabeth River and the lower Chesapeake Bay, near Norfolk, Virginia, were also low (2 to 15  $\mu\text{mol g chl a}^{-1}$ ; unpubl. data). While the group of algae examined in this study may not necessarily be representative of coastal assemblages, the lower concentrations in the field as compared to the laboratory data (as well as the lower near-shore and estuarine concentrations compared to the more oceanic measurements) could be due to some nutrient limitation combined with some stress that depletes glutathione. Measurements of phytochelatin in the field are also typically lower than those in the laboratory (Ahner et al. 1997), but it remains to be determined whether this is a direct result of the lower glutathione concentrations. Concentrations of particulate  $\gamma$ -glu-cys were significantly higher than glutathione in Galveston Bay (Tang et al. 2000). In the laboratory cultures, this was only found when the cultures were challenged with metals.

Several recent research papers have suggested that glutathione may be a fairly abundant ligand in surface seawater (Le Gall & van den Berg 1998, Leal et al. 1999, Tang et al. 2000). Le Gall & van den Berg (1998) measured up to 15 nM glutathione in coastal surface water using an electrochemical technique, and Tang et al. (2000) measured up to 6 nM in Galveston Bay using a sensitive HPLC method. There does appear to be somewhat of a conundrum with respect to the source of this dissolved glutathione. Particulate concentrations (normalized to volume) are less than those measured in the dissolved phase (Tang et al. 2000) and, even using the high intracellular concentrations measured in this paper and conservative estimates of turnover rates, it is difficult to predict such high concentrations. Further work should be done to determine the chemical and biological stability of these compounds in the field. It is possible that we are underestimating the source terms

in our calculations. Indeed, very high rates of glutathione efflux must be invoked to explain the rapid increase in dissolved ligand concentrations observed by Vasconcelos & Leal (2001) in response to Cu additions to *Emiliania huxleyi* cultures that were attributed to a glutathione-like compound.

In conclusion, low molecular weight thiols are very important biochemicals and, through laboratory studies, we have come one step closer to understanding the potential influence of some environmental variables on their intracellular concentrations.

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