

Interactions between Cd, Cu, and Zn Influence Particulate Phytochelatin Concentrations in Marine Phytoplankton: Laboratory Results and Preliminary Field Data

LIPING WEI,[†] JOHN R. DONAT,[‡]
GARY FONES,[§] AND BETH A. AHNER^{*†}

*Biological and Environmental Engineering,
Cornell University, Ithaca, New York 14853,
Department of Chemistry and Biochemistry,
Old Dominion University, Norfolk, Virginia 23529, and
Department of Chemistry, Woods Hole Oceanographic
Institution, Woods Hole, Massachusetts 02543*

The effect of metal interactions on phytochelatin production by marine phytoplankton has received little attention but yet is critical to understanding the biochemical production of this potentially important metal-binding ligand in the field. Cd, Cu, and Zn additions were made singly and in combination to three species of laboratory cultures and to a natural algal assemblage from pristine coastal seawater. In the laboratory cultures intracellular phytochelatin varied with metal exposure and demonstrated metal- and concentration-dependent synergisms and antagonisms. Most notably, the addition of all three metals together greatly suppressed phytochelatin production in all cultures. Particulate phytochelatin was also measured at two field sites. In the field, phytochelatin production is related to ambient Cd, Cu, and Zn levels, and the deviations from the dose–response relationship are potentially explained by metal interactions similar to those observed in the laboratory cultures. Though particulate glutathione concentrations were very low in some field samples, it did not appear to limit phytochelatin production. Particulate phytochelatin concentrations in samples from both field sites were very similar to those measured in the laboratory cultures when exposed to all three metals together, and thus phytochelatin levels in the field may be regulated by the interaction of Cd, Cu, and Zn.

Introduction

Human activities have greatly increased heavy metal concentrations in estuarine and coastal waters through agricultural, industrial, or domestic discharges (1). In particular, cadmium, copper, and zinc are widespread contaminants, which are toxic to marine phytoplankton at high concentrations. Organic complexation of trace metals in seawater and

the antagonistic interaction between metals with respect to uptake (2–4) make it difficult to predict ecological damage or even determine the involvement of trace metals once ecological changes have occurred.

Phytochelatin is a metal-binding peptide, $[(\gamma\text{-glu-cys})_n\text{-gly}]$ where $n = 2\text{--}11$ (5), produced by eukaryotic marine phytoplankton in response to metal exposure (6–8) and may function as bioindicators of metal contamination in coastal seawater (9, 10). They are synthesized by the constitutive enzyme phytochelatin synthase which catalyzes the transfer of $\gamma\text{-glu-cys}$ from glutathione ($\gamma\text{-glu-cys-gly}$) to another glutathione molecule to form phytochelatin $n = 2$, or to $(\gamma\text{-glu-cys})_n\text{-gly}$ to form phytochelatin chains of length $n + 1$ (11). Environmentally relevant levels of Cd, Cu, and Zn induce production of short chain phytochelatin in laboratory cultures of marine algae (6, 7), and Cu has been linked to changes in phytochelatin concentrations in one field study (9). Glutathione also serves as a metal chelator in the cytoplasm (12), and glutathione synthesis has been linked to metal stress in higher plants (13). While glutathione concentrations do not vary with metal stress in algal cultures (14), they appear to be highly variable in the field (15, 16) where it has been proposed to limit phytochelatin synthesis (9).

It has been shown that metal interactions are likely important to phytochelatin production in the field (9), but the production of particulate phytochelatin and other low molecular weight thiols in response to combinations of Cd, Cu, and Zn has never been comprehensively studied. Here we report the results of our study in which the interactions of these metals have been examined with three algal species over a range of metal concentrations in laboratory experiments and in an incubation experiment with pristine coastal seawater. We also report measurements of particulate phytochelatin and other thiol concentrations at two separate field sites. In one field experiment, we examined the relationship between free Cd, Cu, and Zn ion concentrations and concentrations of particulate phytochelatin and glutathione.

Materials and Methods

Algal Culture Preparation. *Thalassiosira pseudonana* (CCMP 1335; Provasoli-Guillard Center for the Culture of Marine Phytoplankton, West Boothbay Harbor, ME), *Skeletonema costatum* (CCMP1332), and *Dunaliella tertiolecta* (CCMP 1320) 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 minimal medium developed specially for studies of trace metal nutrition and toxicity in marine algae (17). Additions of stock solutions of equimolar Cd-EDTA, Cu-EDTA, and Zn-EDTA were used to increase trace metal concentrations of above standard Aquil levels. Free metal ion concentrations were set as follows (expressed as $\text{pMe} = -\log [\text{Me}^{2+}]$): pCd: 12, 11, 10, and 9; pCu: 12, 11, and 10; and pZn: 8 and 7. Total Me-EDTA additions required for each free metal ion concentration were based on equilibrium calculations made using MINEQL (18) and are listed in Table 1. *T. pseudonana* and *D. tertiolecta* were grown in media containing constant free metal ion concentrations for at least 18 or 19 generations before cells were collected for *chl a* and thiol analysis near the end of exponential growth. Growth was monitored by fluorescence measurements using a Turner Design fluorometer. The growth rates of *T. pseudonana* and *D. tertiolecta* did not vary over 10% when the culture was exposed to individual or combinations of these metals. *S. costatum* did not grow well at high concentrations of added

* Corresponding author phone: (607)255-3199; fax: (607)255-4080; e-mail: baa7@cornell.edu. Corresponding author address: Riley-Robb Hall, Cornell University, Biological and Environmental Engineering, Ithaca, NY 14850.

[†] Cornell University.

[‡] Old Dominion University.

[§] Woods Hole Oceanographic Institution.

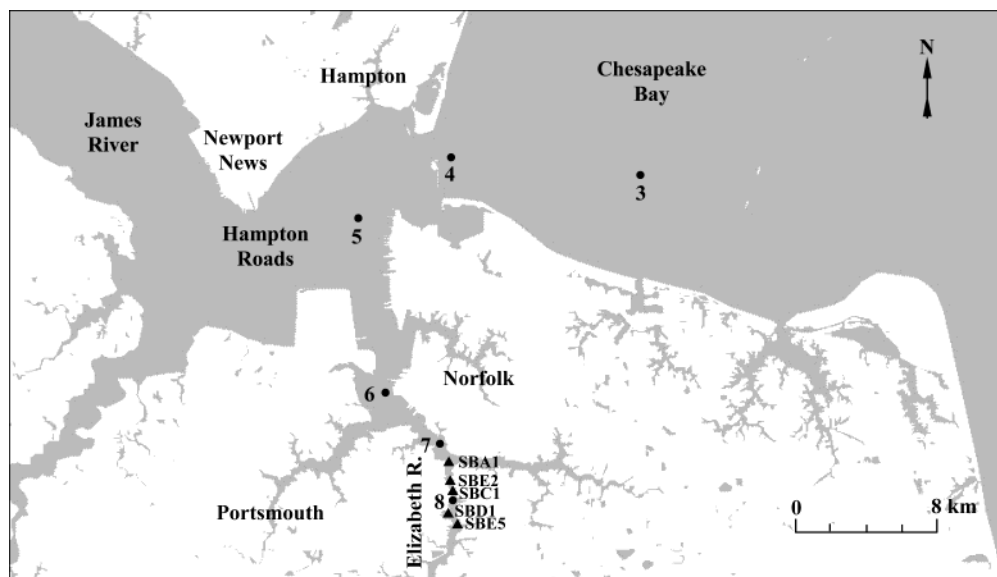


FIGURE 1. Sampling stations (Stn 3–8) in the Elizabeth River estuary during May 2000 and sampling stations in the southern end of the Elizabeth River estuary during April 2002 (SBA1, SBE2, SBC1, SBD1, SBE5). Station SBD4 is located about 4 km south of SBE5 and does not appear on the map.

TABLE 1. Concentrations (μM) of Equimolar Metal–EDTA Added To Achieve the Listed Free Metal Concentrations in Aquil^a

		pMe =					
		12	11	10	9	8	7
Cd	Cd-EDTA	0.03	0.3	3	30		
	TM EDTA	100	100	100	100		
Cu	Cu-EDTA	1.2	12	120	120		
	TM EDTA	100	100	100	100		
Zn	Zn-EDTA					60	60
	TM EDTA					100	100

^a pMe is equivalent to $-\log[\text{Me}^{2+}]$. TM EDTA indicates the concentration of EDTA present in the medium due to the base trace metal mixture. Compared to standard Aquil medium, 10-fold less trace metal mix is used for the highest Cu and Zn treatments. Total EDTA in the medium is the sum of the TM EDTA and that added to elevate the concentration of Cd, Cu, or Zn.

metals. Therefore, metals were added to exponentially growing control cultures for short-term incubations (48 h) before cells were harvested for *chl a* and thiol analysis.

Field Sampling for Particulate Thiols. Water samples were collected from different stations in the Elizabeth River estuary, VA (Figure 1), during May 2000 at 1-m depth with a peristaltic pump and acid cleaned Teflon tubing (19), and from Eel Pond in Woods Hole, MA, during July 2001 at the surface (0.1–0.5 m) with a Teflon bottle mounted on a pole sampler (20). In April 2002, several additional samples were collected from the Elizabeth River estuary (near stations 8 and 7, Figure 1). Samples were returned to the laboratory and dispensed into acid-cleaned polycarbonate bottles, and the particulate fraction was collected within 4 h by gentle filtration (<5 psi) onto 25 mm Whatman GF/F filters (nominal retention 0.7 μm). Filters were changed before significant clogging was observed (roughly 200–500 mL per filter). The filters were immediately stored in liquid nitrogen for later analysis of total *chl a*, particulate phytochelatin, and other low molecular weight thiols.

Incubation of Natural Seawater with Metal Additions. Surface seawater was collected using acid-cleaned polycarbonate bottles from Vineyard Sound, Woods Hole, MA, during July 2001. Duplicate 2-L seawater samples were incubated with additions of 500 pM CdCl_2 , 50 nM CuSO_4 , or 200 nM

ZnSO_4 , or a combination thereof. Seawater was incubated immediately under constant light flux of 40 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ at 20 °C. After a 24-h incubation, samples for later analysis of *chl a* and particulate phytochelatin and other low molecular weight thiols (i.e. cysteine, γ -glu-cys, and glutathione) were collected as described above.

***Chl a* Measurement.** *Chl a* was extracted in 90:10 acetone:water (21) or 45:45:10 DMSO:acetone:water with 0.1% diethylamine added as an antioxidant at 4 °C in the dark (22). Fluorescence was measured on a Turner Design 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. The first set of Elizabeth River samples were extracted with acetone–water, and the subsequent samples were extracted with DMSO–acetone–water. The DMSO protocol takes less time and is more efficient for some algae, and the extracted *chl a* is stable for a longer period of time (S. Huntsman, personal communication). In the first set of Elizabeth River samples, zeaxanthin, a diagnostic accessory pigment for prokaryotic phytoplankton, was determined by HPLC (23, 24). Concentrations of zeaxanthin were very low; therefore, we assumed that the contribution to total *chl a* from prokaryotic species (which do not synthesize phytochelatin) was minimal. Total *chl a* was used to normalize thiol concentrations in the laboratory and field.

Analysis of Low Molecular Weight Thiols. To measure particulate thiols, samples were denatured and homogenized in 10 mM methanesulfonic acid (MSA) as described by Ahner et al. (14, 25). For field samples, it was sometimes necessary to combine more than one filter in a single analysis to obtain sufficient biomass. The MSA extract was retained for derivatization with the fluorescent tag monobromobimane (25–27). HPLC was then used to separate and quantify the low molecular weight thiols. HPLC analyses were performed on a Beckman HPLC equipped with a Gilson 121 filter fluorometer (310–410 nm excitation, 475–650 nm emission). Three separate HPLC columns, buffers, and elution gradients were used for analyses (see Table 2 for details). Methods A and B were used initially and subsequently an improved method, capable of measuring all thiols in one run, replaced these two methods (method C). Each method was independently calibrated with known concentrations of individual thiols. Only the concentrations of the $n = 2$

TABLE 2. HPLC Methods Used for the Analysis of Phytochelatin and Other Low Molecular Weight Thiols

	method A	method B	method C			
analytes	phytochelatin	glutathione ^a	cysteine, γ -glu-cys, glutathione, phytochelatin			
column	Alltech C18 Solvent Miser (2.1 \times 250 mm)	Alltech C8 Solvent Miser (2.1 \times 250 mm)	Supelco Discovery RP Amide-C16 (4.6 \times 250 mm)			
flow rate	0.2 mL min ⁻¹	0.2 mL min ⁻¹	1 mL min ⁻¹			
HPLC solvents						
A	18% acetonitrile, 0.25% acetate buffer, pH 4.2, 0.64 mM tetraoctylammonium bromide	42 mM acetate buffer pH 4.5	25 mM KH ₂ PO ₄ /H ₃ PO ₄ pH 3.2			
B	acetonitrile	acetonitrile	acetonitrile			
	time (min)	% B	time (min)	% B	time (min)	% B
time program (linear gradients were used throughout)	0	0	0	0	0	8
	14	39	5	24.6	2	8
	42	45	10	0	12	12.5
	50	83	25	70	14.7	16.4
	52	0	27	0	25	18.4
	60	0	37	0	31	40
					34	80
					36	80
					37	8
					40	8

^a This method could not separate glutathione from cysteine and γ -glu-cys.

TABLE 3. Total Dissolved and Free Metal Ion Concentrations (\pm SD) of Mn, Cd, Cu, and Zn in the Elizabeth River Estuary during May 2000, and Eel Pond (\pm SD) during July 2001^a

site	Mn	Cd		Cu		Zn	
	total dissolved (nM)	total dissolved (pM)	pCd	total dissolved (nM)	pCu	total dissolved (nM)	pZn
Elizabeth River Estuary, May 2000							
Stn 8	789.2 \pm 61	485.6 \pm 20.8	10.5	50.1 \pm 1.2	11.7	165.0 \pm 22	6.9
Stn 7	692.6 \pm 70	540.3 \pm 5.8	10.5	36.7 \pm 0.8	12.3	128.8 \pm 4.4	7.2
Stn 6	353.3 \pm 43	562.7 \pm 7.4	10.5	29.6 \pm 0.1	12.5	129.6 \pm 0.3	7.0
Stn 5	25.0 \pm 1.7	151.6 \pm 2.0	11.5	9.4 \pm 0.7	13.4	14.0 \pm 1.2	10.2
Stn 4	18.8 \pm 3.2	117.8 \pm 0.3	11.8	9.1 \pm 0.5	13.3	14.9 \pm 0.9	10.2
Stn 3	28.6 \pm 4.2	94.0 \pm 3.4	11.7	5.9 \pm 0.4	13.3	4.9 \pm 0.1	10.3
Eel Pond, July 2001							
Redfield				53	10.0		
Swope	201.3 \pm 6	bd ^b		66	9.6	115.3 \pm 3.6	
WHOI Dock				70	9.7		
Dyer Dock	75.9 \pm 2.3 ^c	230.2 \pm 9.5 ^c		10.3	10.83	49.4 \pm 0.8 ^c	

^a Free ion concentrations of Cd, Cu, and Zn are reported as pMe (pMe = $-\log$ [Me²⁺]). ^b bd (below detection limit). ^c Measured exchangeable metal via DGT and assumed to be equivalent to total dissolved at this site (see text).

phytochelatin oligomer are reported since $n = 2$ is the dominant phytochelatin oligomer in laboratory cultures except at high Cd concentrations and $n = 3$ and 4 are below detection in the field samples. Replication of experimental data points varied from experiment to experiment; therefore, we have specified the number of replicates in the caption of each figure.

Total Dissolved and Free Metal Ion Measurements. Several different techniques for determining free and total metal ion concentrations were used depending on the particular metal and the expertise of the collaborator available to do analyses at a particular site.

In the Elizabeth River estuary during May 2000, free Cd²⁺, Cu²⁺, and Zn²⁺ ion concentrations at each sampling station were calculated using the equilibrium speciation computer program MINEQL (18) from the concentration of metal complexing ligands and their conditional stability constants determined for Cd and Zn using anodic stripping voltammetry (ASV (28, 29)) and for Cu using competitive ligand exchange-adsorptive cathodic stripping voltammetry (CLE-ACSV) with salicylalidoxime (SA) as the competitive ligand

(19, 29). Total dissolved metal concentrations were determined using the complexation/extraction method of ref 30 as modified by Statham (31). Metal analyses were not performed on samples collected during April 2002.

In Eel Pond, free Cu²⁺ concentrations were measured using CLE-ACSV with benzoylacetone (BZAC) as the competitive ligand, a method that yields results comparable to those obtained with SA (32). Total dissolved copper was determined by CLE-ACSV with BZAC after UV-oxidation, acidification, and pH adjustment to ~ 8 with isothermal distilled ammonia. Total Mn, Cd, and Zn in the sample collected from Swope station of Eel Pond were measured using ICP-MS on filtered seawater. The technique of diffusive gradient in thin-films (DGT), followed by elution with nitric acid and ICP-MS measurement (33), was used to measure exchangeable Mn, Cd, and Zn for the samples collected from the Dyer Dock station. These measurements have been reported as totals in Table 3 because virtually all soluble Mn in surface seawater is Mn²⁺ (34) and, at this site, total Zn is very close to exchangeable Zn (James Moffett, personal communication). In the case of Cd, it has been shown that

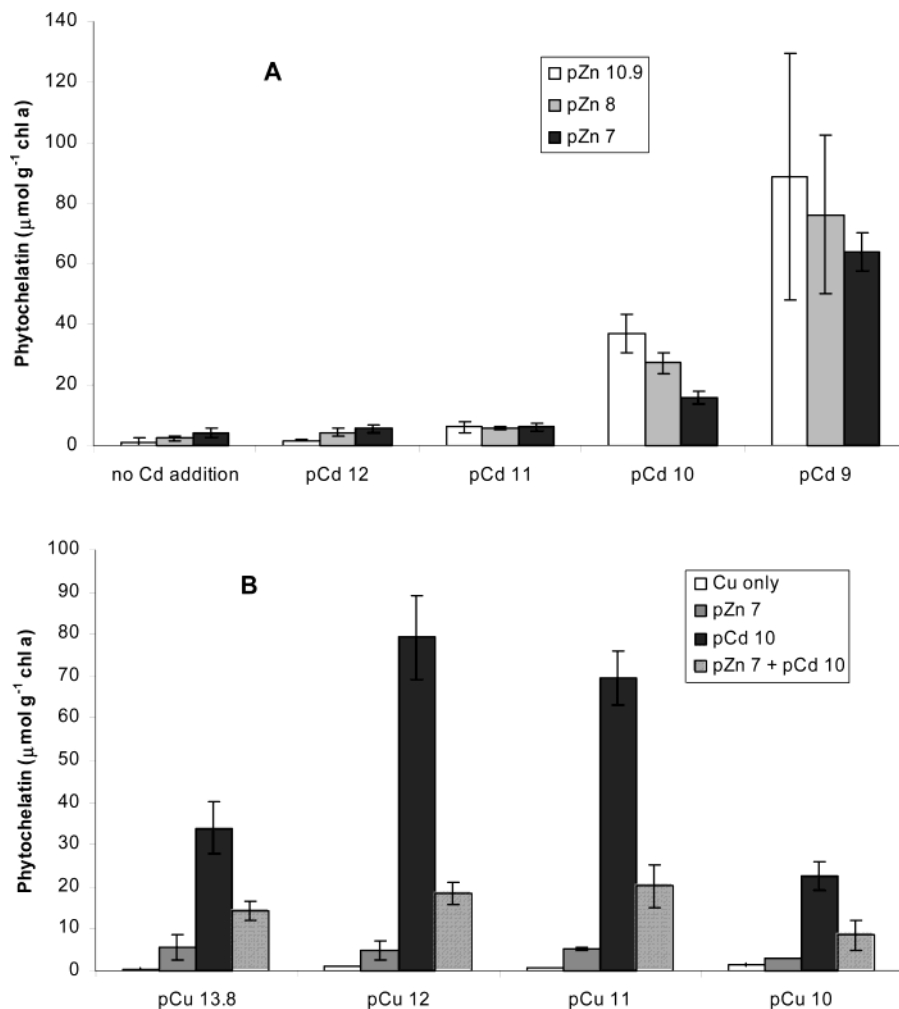


FIGURE 2. Phytochelatin ($n = 2$) production by *Thalassiosira pseudonana* (A) cultured under conditions of increasing free Cd^{2+} concentrations and three levels of free Zn^{2+} , where pZn 10.9 is the background concentration in AQUIL, and (B) under conditions of increasing concentrations of free Cu^{2+} , with or without Cd and/or Zn addition to a final concentration of pCd 10 and pZn 7. Error bars represent the standard deviation of three independent samples.

in the presence of natural ligands (humic and fulvic acids), DGT measures about 90% of the total dissolved Cd (35).

Results and Discussion

Phytochelatin Production in Metal-Exposed Laboratory Cultures. To determine the effect of multiple metals on phytochelatin production, we measured particulate phytochelatin and other low molecular weight thiols in algae that were exposed to Cd, Cu, and Zn individually and in combination. In cultures of *T. pseudonana* exposed to long-term constant free metal ion concentrations, phytochelatin production increased with increasing free Cd^{2+} at all three Zn concentrations examined (Figure 2A). In addition, increasing free Zn^{2+} alone also caused slight increases in phytochelatin production in the cultures to which no Cd had been added and in those with pCd 12. At pCd 11, we begin to see the effects of a Zn/Cd antagonism with respect to phytochelatin production, since no increase was observed with Zn addition. At pCd 10, increasing Zn^{2+} resulted in significant decreases in phytochelatin (up to 50% at the highest Zn concentration), whereas at pCd 9, somewhat less antagonism was observed. Thus, the effects of Cd and Zn may be additive, but above a certain threshold concentration of Cd, they become antagonistic with respect to phytochelatin production, and the extent of the antagonism is dependent on both Cd and Zn levels.

Cu alone did not induce production of large amounts of phytochelatin in *T. pseudonana*, but Cu interacted with Cd and Zn in both antagonistic and synergistic ways to influence phytochelatin production (Figure 2B). At all free Cu^{2+} concentrations, adding a high concentration of free Zn^{2+} (pZn 7) resulted in small increases in phytochelatin; at the highest free Cu^{2+} concentration (pCu 10), a slight antagonistic effect between Cu and Zn was observed (i.e. a slight decrease in phytochelatin concentration as compared to those at lower Cu with the same Zn). The addition of Cd (pCd 10) to the Cu-treated cultures resulted in large though variable increases in phytochelatin production. Synergistic effects were observed between Cd and moderate concentrations of Cu (pCu 12 and 11): phytochelatin concentrations were greater than the sum of those when the cultures were exposed to the metals individually. However, when the free Cu^{2+} concentration was greater (pCu 10), an antagonistic effect was observed: phytochelatin concentrations were less than the sum of those when the cultures were exposed to the metals individually. Adding all three metals simultaneously to the cultures significantly decreased phytochelatin production as compared to all of the cultures containing both Cd and Cu, and phytochelatin production was lowest at the highest free Cu^{2+} concentration.

Additional long-term experiments were performed with *D. tertiolecta*, a green alga, using the concentrations of free

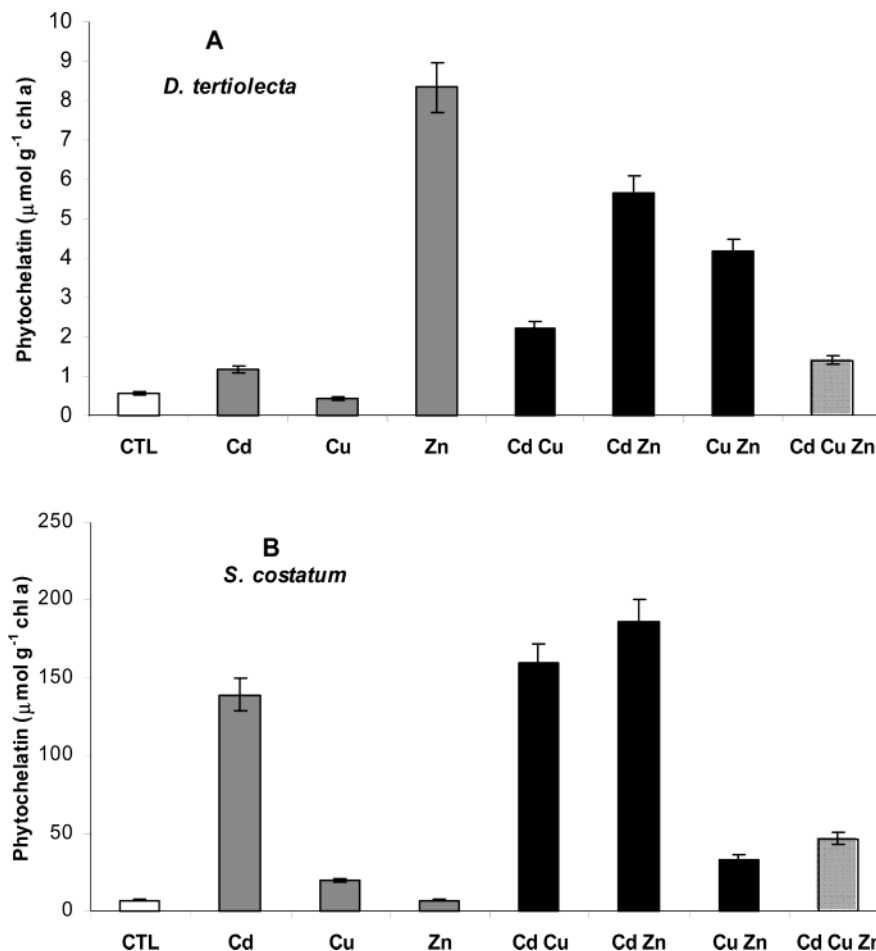


FIGURE 3. Phytochelatin ($n = 2$) production by *Dunaliella tertiolecta* cultured under specified conditions of Cd, Cu, and Zn exposure (A) and *Skeletonema costatum* upon short-term exposure to the same set of metals (B). For error bars, we have used a standard deviation of 8%, representative of our average analytical error on duplicate samples.

Cd^{2+} , Cu^{2+} , and Zn^{2+} that resulted in the most significant interactions in the previous experiments: pCd 10, pCu 10, and pZn 7. In general, phytochelatin concentrations (normalized to *chl a*) are much lower in *D. tertiolecta* compared to *T. pseudonana* (Figure 3A). When *D. tertiolecta* cells were exposed to the individual metals, Zn stimulated the most phytochelatin production and Cu induced the least (Figure 3A). No antagonism or synergism was observed when Cd and Cu were added together, whereas significant decreases in Zn-induced phytochelatin were generated when Cd or Cu was also added. The addition of all three metals together further suppressed phytochelatin production. Phytochelatin oligomers of length $n = 3$ and 4 were also synthesized in *D. tertiolecta* (data not shown) and showed a similar pattern of production with exposure to the various metals.

If we generalize our results from the long-term experiments with *T. pseudonana* and *D. tertiolecta* exposed to pCd 10, pCu 10, and pZn 7, it appears that when phytochelatin production is stimulated predominantly by one metal, the addition of another metal suppresses phytochelatin synthesis, and addition of all three metals suppresses it further. Furthermore, the result of the experiments with *T. pseudonana* at different concentrations of free Cd^{2+} , Cu^{2+} , and Zn^{2+} suggests that the interaction between pairs of metals is strongly dependent on their relative concentrations, and most likely, there exists a threshold for different interactive effects with respect to phytochelatin production.

When *S. costatum* was exposed during short-term incubations (48 h) to Cd, Cu, and Zn at the same concentrations used in the *D. tertiolecta* experiments, more phytochelatin

was produced in response to Cd than to Cu or Zn (Figure 3B). While it was not our intention to do a comprehensive comparison between long-term and short-term exposure, it is interesting to note that in these experiments when the metals were added in pairs, no antagonistic or synergistic effects on phytochelatin concentrations were observed; concentrations increased additively. However, the combination of all three metals resulted in a large reduction of phytochelatin production relative to all other cultures containing Cd, similar to that observed in the long-term experiments with *T. pseudonana* and *D. tertiolecta*. The lack of an antagonistic or synergistic effect on phytochelatin concentrations between pairs of metals in *S. costatum* may simply be the result of the relatively short-term metal exposure or that the cells were under extreme metal stress compared to the other algal species (recall that this species was not able to grow at these free Cd^{2+} , Cu^{2+} , and Zn^{2+} concentrations which is why we did these short-term experiments). The cell's necessary feedback regulation on metal uptake (36) may have been impaired or not have had time to develop. It is worth noting that the *S. costatum* isolate used in this study produces much less phytochelatin upon exposure to Cu and Zn than the one previously examined by Ahner et al. (9). It is possible that these two strains, obtained from different sources, are actually different species since morphological similarities have historically been used for classification.

The antagonisms observed here are likely due to competition between metals for uptake sites. Sunda and Huntsman (3) demonstrated that Cd enters *T. pseudonana*

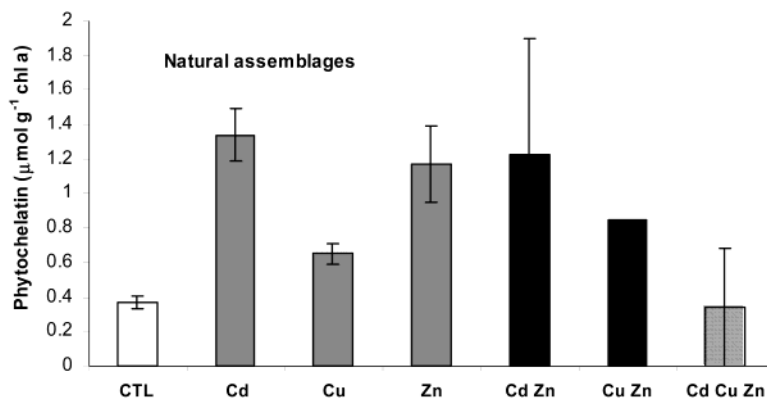


FIGURE 4. Concentrations of particulate phytochelatin ($n = 2$) in a natural phytoplankton assemblage after 24 h of metal exposure. Incubations with 500 pM CdCl₂, 50 nM CuSO₄, or 200 nM ZnSO₄ and in combination were done with natural seawater collected from Vineyard Sound, July 2001. No metals were added in the control incubation. Error bars indicate the high and low values of duplicate measurements on individual incubations. No duplicate was available for the Cu/Zn incubation.

cells through either the Zn or Mn ion channels; therefore, the presence of high free Zn²⁺ or high free Mn²⁺ can decrease Cd uptake and, ultimately, its toxicity. Lower phytochelatin production in response to Cd in the presence of high Zn (Figure 2A) can thus be understood. We have also observed a 50% reduction in phytochelatin production in *T. pseudonana* in response to pCd 10 in the presence of high free Mn²⁺ (pMn 6.3), compared to pMn 8.3 in control cultures (unpublished data). Likewise, other studies have shown that Cu toxicity is ameliorated by increasing concentrations of free Zn²⁺ (4) and free Mn²⁺ (2) because of a competitive uptake mechanism; thus, the antagonism that we observed between high Zn (or Mn) and Cu and phytochelatin production could be expected. The antagonism seen between high Cd and high Cu (pCd 10 and pCu 10 in *T. pseudonana*) in this study is likely related to these same competitive uptake mechanisms, since both metals must compete with each other for the Zn and Mn transport sites.

Synergistic effects are more difficult to explain. One explanation is that at intermediate concentrations of Cd and Cu, these metals did not inhibit uptake of each other but did inhibit the uptake of Zn or Mn. The cell, potentially experiencing a Zn or Mn limitation under these conditions, makes more Zn or Mn transporters, which allows for greater intracellular accumulation of Cd and Cu and thus greater phytochelatin concentrations. It is also possible that there are synergistic effects of intracellular metals on the expression of phytochelatin synthase.

Phytochelatin Production in Metal-Exposed Natural Assemblages. To examine the response of a natural algal assemblage, metal additions were made to short-term incubations of pristine unfiltered coastal seawater. Phytochelatin concentrations ranged from 0.3 to 1.3 µmol g⁻¹ chl a and varied with metal exposure (Figure 4). Compared to the control incubation in which no metal was added, the addition of Cd, Cu, or Zn all resulted in an increase in phytochelatin production by 3.6, 1.8, and 3.2 times, respectively. Metal combinations of Cd + Zn and Cu + Zn caused a similar 2–3-fold increase, but given the variability of the duplicates (for Cd + Zn) and the lack of a duplicate data point (for Cu + Zn), it is not possible to confidently evaluate whether any antagonism occurred. The addition of all three metals together resulted in a phytochelatin concentration that was statistically indistinguishable from that of the control ($p > 0.05$). Thus, the natural phytoplankton assemblage appears to be subject to the same antagonism (i.e. suppression of phytochelatin production) resulting from the combination of Cd, Cu, and Zn that occurs in the laboratory cultures.

Phytochelatin Production in the Field. Phytochelatin concentrations were measured during two separate trips to the Elizabeth River estuary and in Eel Pond. The range of concentrations measured compare well with other coastal studies (9, 25) and are also remarkably similar to those measured in cultures that have been exposed simultaneously to high concentrations of Cd, Cu, and Zn. Concurrent trace metal measurements were made during the first trip to the Elizabeth River and in Eel Pond.

A strong gradient in the total dissolved concentration of Cd, Cu, and Zn was observed in the Elizabeth River estuary during May 2000, with high concentrations in the Elizabeth River and lower concentrations in Hampton Roads and further out into Chesapeake Bay (Figure 1 and Table 3). Phytochelatin concentrations ranged from 1.1 to 18.6 µmol g⁻¹ chl a (Table 4). Consistent with the observation in the cultures, phytochelatin production increased in general with increasing free metal ion concentration. A positive relationship between particulate phytochelatin and the log of the free Cu²⁺ ion concentration was observed (for comparative purposes a linear relationship is shown in the figure, $r^2 = 0.76$, $n = 6$, Figure 5A); there were also significant correlations between phytochelatin and Cd²⁺ ($r^2 = 0.49$, $n = 6$, Figure 5A) and Zn²⁺ ($r^2 = 0.47$, $n = 6$, Figure 5B). There was, in addition, somewhat of a relationship between phytochelatin and total metal concentrations, which is likely due to the strong correlation between total metal and free metal ion concentrations at this field site (Table 3). The high concentrations of Cd²⁺, Cu²⁺, and Zn²⁺ measured at stations 6, 7, and 8 are similar to those used in the laboratory experiments. Given what is known about the relative abilities of Cd, Cu, and Zn to induce phytochelatin from this and other studies, Cd is likely the major inducer of intracellular phytochelatin at this site. All of the phytochelatin concentrations in the metal contaminated sites are depressed relative to what we might expect on the basis of the individual metal concentrations (especially if we assume diatom domination of the algal assemblage which has been the historical situation (37)).

It is notable that although Cd, Cu, and Zn concentrations were similar in the more polluted stations (stations 6, 7, and 8), phytochelatin was nearly an order of magnitude lower at station 6 compared to the concentrations measured at stations 7 and 8 (Table 4). Although it is not possible to exclude other explanations (as noted below), such differences may be explained by the metal interactions we observed in the laboratory cultures. The synergism between Cu and Cd that occurred in *T. pseudonana* at pCd 10 and at intermediate concentrations of free Cu²⁺ (pCu 12 and 11) may have elevated the phytochelatin concentration at stations 7 and 8, while at

TABLE 4. Ancillary Data, *chl a*, and Particulate Thiol Concentrations for Samples Collected in the Elizabeth River Estuary and Eel Pond, Woods Hole^a

site	pH	temp (°C)	salinity	NO _x (μmol N/L)	<i>chl a</i> (μg L ⁻¹)	PC (μmol g ⁻¹ <i>chl a</i>)	GSH (μmol g ⁻¹ <i>chl a</i>)	PC:total
Elizabeth River Estuary, May 9, 2000								
Stn 8	7.4	21.1	16.0	10.0	3.3	18.6 ± 0.3	11 ± 0.7	0.77
Stn 7	7.5	20.7	16.8	5.8	6.4	15.3 ± 2.0	4 ± 0.9	0.8
Stn 6	7.7	20.2	17.1	0.80	7.2	2.1 ± 0.2	4 ± 0.4	0.3
Stn 5	8.0	19.9	17.3	bd ^b	9.8	1.1 ± 0.1	1 ± 0.1	0.4
Stn 4	8.1	19.1	19.5	bd ^b	10.0	1.4 ± 0.1	1 ± 0.01	0.4
Stn 3	8.1	17.7	22.1	bd ^b	3.8	3.6 ± 0.1	4 ± 0.2	0.6
Elizabeth River Estuary, April 18, 2002								
SBD4	7.5	22.4	16.1	10.0	13.1	1.4, bd ^b	145 ± 50	
SBE5	6.3	26.7	18.9	10.8	6.7	1.0, bd ^b	127 ± 6	
SBD1	7.4	23.5	18.5	8.4	11.0	1.5, 4.3	118 ± 11	0.046
SBC1	7.7	20.8	19.7	9.6	10.9	0.3, 2.5	178 ± 18	0.016
SBE2	7.6	20.1	20.2	10.1	9.6	2.0, 1.8	155 ± 7	0.024
SBA1	8.0	20.2	20.4	8.7	22.1	bd, ^b 0.8	108 ± 7	
Eel Pond, Woods Hole, July 2001								
Redfield	nm ^c	nm ^c	nm ^c	nm ^c	6.7	0.7 ± 0.03	74 ± 3.2	0.02
Swope	nm ^c	nm ^c	nm ^c	nm ^c	6.4	1.0 ± 0.4	83 ± 14.5	0.02
WHOI Dock	nm ^c	nm ^c	nm ^c	nm ^c	5.4	1.4 ± 0.1	82 ± 2.7	0.03
Dyer Dock	nm ^c	nm ^c	nm ^c	nm ^c	1.9	4.5 ± 0.4	33 ± 10.3	0.19

^a For particulate phytochelatin (PC, *n* = 2) and glutathione (GSH) we report the average and error (representing half the distance between the high and low values of two independent measurements). The molar ratio of phytochelatin thiol to total thiol (PC:total) is calculated as 2*[PC]/([cysteine] + [γ-glu-cys] + [GSH] + 2*[PC]). Longer oligomers of phytochelatin were not detected in these samples. Ancillary data for the Elizabeth River Estuary was collected by W. Sunda in May 2000 and by the Water Quality Laboratory at Old Dominion University in April 2002. NO_x represents the sum of the nitrate and nitrite concentrations. ^b bd: below detection limit for phytochelatin (<0.1 μmol g⁻¹ *chl a*). ^c nm: not measured.

station 6 the free Cu²⁺ concentration, though only marginally lower than at station 7, may not have been high enough to result in an interaction (i.e. it may be lower than the threshold concentration). In addition, an antagonism between Cd and Zn may have occurred at station 6. While concentrations of free Zn²⁺ were not significantly higher at station 6 than at stations 7 and 8, independent experiments performed on water collected at the same time as the samples for phytochelatin analysis revealed that the algal assemblage from station 6 had the highest Zn:C assimilation ratio measured during short-term incubations with radiotracers (W. Sunda, personal communication). While we have no mechanistic explanation for this result, it does suggest that the relatively high free Zn²⁺ may be suppressing phytochelatin production at this particular site.

It is also possible that among these stations the 10-fold variation observed in phytochelatin concentration is due to nonmetal related factors such as species composition (potentially significant given the heterogeneous environment of the estuary, Table 4) or differences in nutrient availability. The nitrate concentration at station 6 was significantly lower than at stations 7 or 8 (Table 4), and there is some evidence that nitrogen limitation can limit thiol production in marine algae (38).

During our return trip to the Elizabeth River in April of 2002, when sampling was focused on the region near stations 7 and 8, the phytochelatin was uniformly low (Table 4). The higher salinity measured in the same reach of the river as compared to May 2000 (Figure 1 and Table 4) suggests that nutrient and/or trace metal concentrations may have been significantly different at this time. In addition, since we sampled 3 weeks earlier in the season, we likely encountered the spring bloom in a different stage of development.

Particulate phytochelatin was also measured at several stations in and out of Eel Pond, a small harbor in Woods Hole, MA. Inside the pond there were high levels of total dissolved Zn and Cu and as well as high levels of free Cu²⁺, but Cd (only measured at the Swope station) was below detection. Outside the pond at the Dyer Dock station, total dissolved Cu and Zn and free Cu²⁺ were much lower, but

total dissolved Cd was quite high (Table 3). Phytochelatin concentrations were 4–6 times higher outside the pond than inside (Table 4). In a previous study, the opposite situation was found, and it was concluded that the high Cu in the pond was inducing phytochelatin production (9). However, Cu is not generally a strong inducer of phytochelatin production, and it is more likely that a Cu/Zn antagonism (analogous to the antagonism observed in *T. pseudonana* when exposed to pCu 10 and pZn 7) caused the very low phytochelatin concentration reported here, while outside the pond at Dyer Dock, the free Cu²⁺ concentration was not above the threshold needed for antagonism. Alternatively, the slightly higher phytochelatin measured at Dyer Dock could also be simply a result of the higher concentration of Cd as compared to inside the pond. In past experiments (9), the total phytochelatin concentrations were also much higher, suggesting a significant shift in community composition and/or metal regime during the elapsed time between sampling events (8 years).

Production of Glutathione and Other Low Molecular Weight Thiols in Laboratory Cultures and in the Field. Since glutathione is the main precursor from which phytochelatins are synthesized (11) and other low molecular weight thiols such as cysteine and γ-glu-cys are precursors for glutathione synthesis, we also examined concentrations of these compounds in our experiments. In response to metal exposure, all three laboratory cultures examined maintained relatively constant levels of glutathione and cysteine, while γ-glu-cys varied nearly as much as phytochelatin (Table 5) and followed generally the same pattern of induction under metal stress (data not shown). The main difference between γ-glu-cys and phytochelatin was that the antagonistic effect of adding all three metals was not as great for γ-glu-cys as for phytochelatin. When exposed to metals singly or in combination, the natural phytoplankton assemblage maintained a relatively constant glutathione concentration, similar to laboratory cultures. Both cysteine and γ-glu-cys appeared to increase upon most metal additions, but no significant antagonisms between metals were observed (data range reported in Table 5).

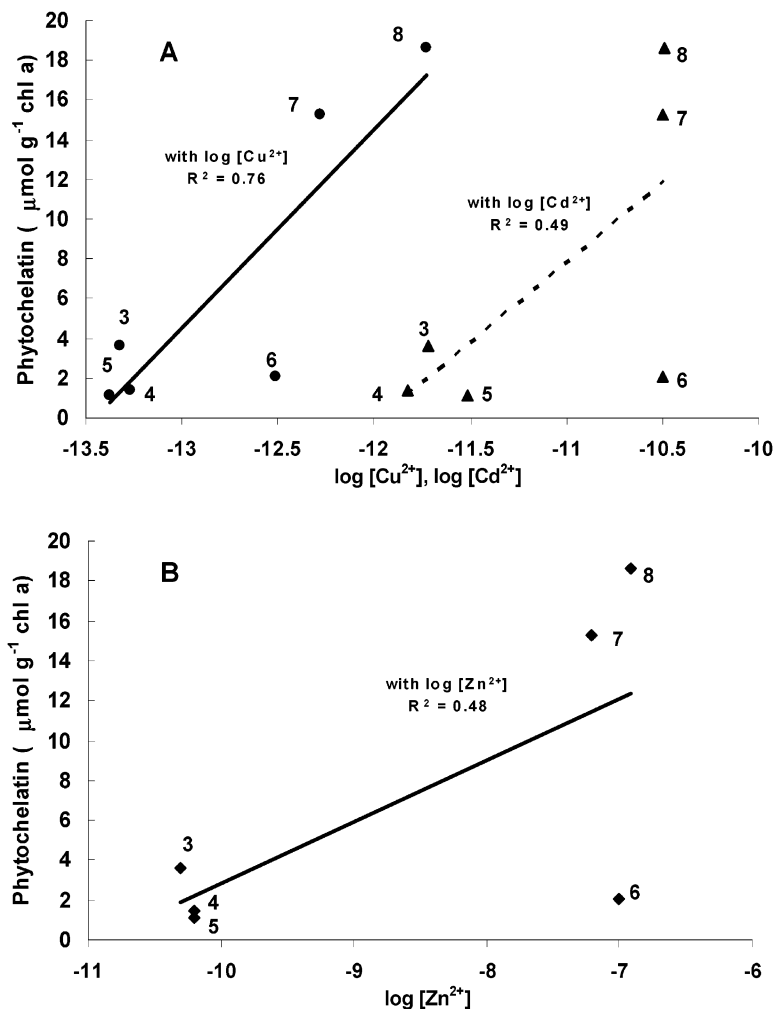


FIGURE 5. Correlations between particulate phytochelatin concentration and log [Me²⁺] (A: log [Cu²⁺] and log [Cd²⁺]; B: log [Zn²⁺]) in the Elizabeth River estuary during May 2000.

TABLE 5. Average (\pm SD) and the Range of Particulate Glutathione (GSH), γ -glu-cys, and Cysteine in the Laboratory Cultures and Field Samples

	GSH ($\mu\text{mol g}^{-1} \text{chl a}$)	γ -glu-cys ($\mu\text{mol g}^{-1} \text{chl a}$)	cysteine ($\mu\text{mol g}^{-1} \text{chl a}$)
<i>T. pseudonana</i>	350 \pm 75	3–52	11 \pm 4
<i>D. tertiolecta</i>	180 \pm 9	0.1–0.8	5 \pm 1.9
<i>S. costatum</i>	364 \pm 23	22–262	29 \pm 7
natural algal assemblage	42.6 \pm 9.8	0.7–2	4.4 \pm 1.3
Elizabeth River estuary, May 2000	1–11 ^a		
Eel pond, July 2001	68 \pm 24	1.1–8	5.2 \pm 1.6
Southern end of the Elizabeth River estuary, April 2002	120 \pm 20	bd ^b –0.9	2.9 \pm 0.4

^a The HPLC method used (Method B) was not able to separate glutathione from γ -glu-cys and cysteine, thus the measurement was the sum of all three thiols. ^b bd: below detection limit ($<0.1 \mu\text{mol g}^{-1} \text{chl a}$).

In the field samples, particulate glutathione varied considerably from site to site (Table 5). In the Elizabeth River estuary during May 2000, glutathione concentrations were very low and ranged from 1 to 11 $\mu\text{mol g}^{-1} \text{chl a}$ and showed less correlation with metals than did phytochelatin. In addition, these concentrations are slightly overestimated because our HPLC method at the time did not separate cysteine and γ -glu-cys from glutathione, but they are likely off only by about 10%, since the amount of cysteine and γ -glu-cys measured in cultures and in field samples is very low compared to glutathione. In April 2002, we returned to sample the most contaminated portion of the estuary (Figure 1) and found 10–100-fold higher glutathione concentrations that again did not vary significantly with station (Table 4).

Glutathione concentrations from Eel Pond generally fell between those measured during the two sampling events in the Elizabeth River estuary (Table 4). The one sample taken outside the pond had significantly less (>2 -fold) glutathione than those taken inside the pond where free Cu²⁺ was high and thus exhibited a pattern opposite that of phytochelatin.

Cysteine and γ -glu-cys were also measured in the field (Table 5). In Eel Pond, γ -glu-cys concentrations and, to a lesser extent, those of cysteine were higher inside the pond where free Cu²⁺ was higher. In the southern end of the Elizabeth River estuary during April 2002, cysteine and γ -glu-cys were low but relatively constant from site to site. In general, cysteine and γ -glu-cys are minor components of the total intracellular low molecular weight thiols, and

cysteine remains constant from station to station, while γ -glu-cys varies. The field results are thus consistent with the laboratory cultures.

Comparing Glutathione and Phytochelatin with Other Field and Laboratory Studies. The range of particulate glutathione concentration measured in the current field studies shows considerable variability (1–178 $\mu\text{mol g}^{-1}$ chl a), ranging nearly 2 orders of magnitude, but is also generally within the range of published measurements. Matrai and Vetter (16) measured glutathione concentrations ranging from 40 to 100 $\mu\text{mol g}^{-1}$ chl a in Saanich Inlet and ~100–200 $\mu\text{mol g}^{-1}$ chl a in surface water from the Southern California Bight, while Tang et al. (15) measured 9 and 66 $\mu\text{mol g}^{-1}$ chl a in upper and lower Galveston Bay, respectively. Glutathione concentrations measured during the two most recent field trips reported here (Elizabeth River, April 2002; Eel Pond, July 2001) ranged from 33 to 178 $\mu\text{mol g}^{-1}$ chl a. However, particulate glutathione concentrations in the Elizabeth River estuary during May 2000 were very low (ranging from 1 to 11 $\mu\text{mol g}^{-1}$ chl a), close to the 9 $\mu\text{mol g}^{-1}$ chl a measured in the upper Galveston Bay (15).

Glutathione concentrations in the field are generally lower than those measured in the laboratory cultures (Table 5). Laboratory cultures in exponential growth maintain a relatively constant level of glutathione (~200–500 $\mu\text{mol g}^{-1}$ chl a) regardless of the amount of phytochelatin synthesized (this study, ref 14). Reduced levels of glutathione have only been observed upon metal addition to some senescent cultures, which may be due to N, P, or other nutrient limitation (14). It appears that high metal concentrations do not result a priori in low glutathione, but nutrient limitations may cause a metal-induced decrease in glutathione. It is possible that the very low glutathione measured in the Elizabeth River estuary during May 2000 was coincident with nutrient limitation following the spring bloom, but when we returned to the same site in April, 2 years later, such limitation had not yet occurred. Other factors in the field, such as oxidative stress or toxicity due to some other contaminant, may also have caused lower glutathione levels.

While field measurements of glutathione were lower than those in the laboratory, the intracellular glutathione level did not appear to limit phytochelatin in the field. In the Elizabeth River estuary during May 2000, glutathione concentrations were extremely low, yet phytochelatin concentrations were comparable to other contaminated field sites [e.g., 1–25 $\mu\text{mol g}^{-1}$ chl a in southeastern New England harbors (9, 25) and 14 $\mu\text{mol g}^{-1}$ chl a and 2 $\mu\text{mol g}^{-1}$ chl a in upper and lower Galveston Bay, respectively, calculated from Figure 6 in ref 15]. Since the rate of phytochelatin production is dependent upon the concentration of both glutathione and the bis-glutathione–metal complex in the cytoplasm (39), the concentration of phytochelatin synthase may be increased to accommodate lower glutathione concentrations. It is also possible that decreases in total glutathione come at the expense of glutathione in the chloroplast or other organelles, and glutathione remains constant in the cytoplasm where phytochelatin synthesis is presumed to occur. In addition we have shown here in our laboratory cultures and field incubation that γ -glu-cys, one of the immediate precursors for glutathione synthesis, varies similarly to phytochelatin in response to metal exposure. It is possible that marine algae utilize an additional pathway for phytochelatin synthesis in which γ -glu-cys is polymerized directly as has been found in yeast (40).

Thiol Ratios. In this study, the particulate thiol concentrations in both laboratory and field samples were normalized to total chl a concentrations. However, cellular chl a concentrations can vary greatly with species, light, nutrient conditions, and sampling season or site and may thus compromise the use of chl a as a measure of algal biomass.

The ratio of phytochelatin thiol to total intracellular low molecular weight thiols might serve as a less compromised parameter to compare laboratory and field data. Since phytochelatin binds metals more efficiently than other low molecular weight thiols and appears to be synthesized specifically for this purpose, a high ratio would indicate a strong bias of intracellular thiol usage for metal detoxification. In the field measurements, this ratio was high in the Elizabeth River estuary (0.3–0.8; Table 4) during May 2000 and at Dyer Dock (0.2) during July 2001, and comparable to ratios observed in the laboratory cultures upon exposure to individual phytochelatin-inducing metals, e.g. cultures of *T. pseudonana* at pCd 11 (0.59) and 10 (0.83), *D. tertiolecta* at pZn 7 (0.20), and *S. costatum* at pCd 9 (0.36). When these algal species are exposed to lower concentrations or combinations of metals, the ratios vary from 0.003 to 0.05, similar to ratios observed in the Elizabeth River estuary during April 2002 and inside Eel Pond during July 2001. It is possible that these ratios may at some point be a useful measure of metal stress in the field, but currently we lack a complete understanding how low molecular weight thiols vary in response to other environmental variables.

In summary, we have presented strong evidence that it is not only the absolute concentrations of free metal ions that control phytochelatin production in the field but also the relative concentration of other competing free metal ions. Antagonistic and synergistic effects of competing free metal ions on phytochelatin production must be considered as most metal-polluted natural systems are contaminated with more than one metal. Phytochelatin concentrations along with ratios of phytochelatin to total low molecular weight thiols may help us to better understand the physiological status of algae with respect to metal stress in the field.

Acknowledgments

Funding for this project was provided by NSF (Grant OCE-0002677 to B.A.A.) and by ONR's Harbor Processes Program (Grant N00014-99-0386 to J.R.D. and D. J. Burdige and N00014-99-1-0093 to J.R.D. and A. S. Gordon). L.W. was supported by a Sage Fellowship from the Cornell University Graduate School. We also thank Cornell undergraduate Franklin Eagan for his help in the laboratory. J.R.D. acknowledges the expertise and assistance of John Consolvo, Christina Dryden, Gonzalo Carrasco, and Matt Brown in determining the total dissolved metal and free metal ion concentrations in the Elizabeth River estuary samples. We are also indebted to Dr. William Sunda from NOAA and Suzanne Doughten from the Water Quality Laboratory at ODU for providing ancillary data from the May 2000 and April 2002 sampling trips, respectively. We also thank our reviewers for helpful comments and suggestions.

Literature Cited

- (1) Oehme, F. W. *Toxicity of heavy metals in the environment*; Marcel Dekker: New York and Basel, 1978.
- (2) Sunda, W. G.; Huntsman, S. A. *Limnol. Oceanogr.* **1983**, *28*, 924–934.
- (3) Sunda, W. G.; Huntsman, S. A. *Environ. Sci. Technol.* **1998**, *32*, 2961–2968.
- (4) Rueter, J. G.; Morel, F. M. M. *Limnol. Oceanogr.* **1981**, *26*, 67–73.
- (5) Grill, E.; Winnacker, E. L.; Zenk, M. H. *Science* **1985**, *230*, 674–676.
- (6) Ahner, B. A.; Kong, S.; Morel, F. M. M. *Limnol. Oceanogr.* **1995**, *40*, 649–657.
- (7) Ahner, B. A.; Morel, F. M. M. *Limnol. Oceanogr.* **1995**, *40*, 658–665.
- (8) Rijstenbil, J. W.; Wijnholds, J. A. *Mar. Biol.* **1996**, *127*, 45–54.
- (9) Ahner, B. A.; Morel, F. M. M.; Moffett, J. W. *Limnol. Oceanogr.* **1997**, *42*, 601–608.
- (10) Pawlik-Skowronska, B. *Aquat. Toxicol.* **2000**, *50*, 221–230.
- (11) Grill, E.; Löffler, S.; Winnacker, E. L.; Zenk, M. H. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6838–6842.

- (12) Rabenstein, D. L. In *Glutathione: chemical, biochemical, and medical aspects*; Dolphin, D., Avramovic, O., Poulson, R., Eds.; Wiley: New York, 1989; pp 147–186.
- (13) Schäfer, H. J.; Haag-Kerwer, A.; Rausch, T. *Plant Mol. Biol.* **1998**, *37*, 87–97.
- (14) Ahner, B. A.; Wei, L. P.; Oleson, J. R.; Ogura, N. *Mar. Ecol. Prog. Ser.* **2002**, *232*, 93–103.
- (15) Tang, D.; Wen, L. S.; Santschi, P. H. *Anal. Chim. Acta* **2000**, *408*, 299–307.
- (16) Matrai, P. A.; Vetter, R. D. *Limnol. Oceanogr.* **1988**, *33*, 624–631.
- (17) Price, N. M.; Harrison, G. I.; Hering, J. G.; Hudson, R. J.; Nirel, P. M. V.; Palenik, B.; Morel, F. M. M. *Biol. Oceanogr.* **1988/1989**, *6*, 443–461.
- (18) Westall, J. C.; Zachary, J. L.; Morel, F. M. M.: *MINEQL: a computer program for the calculation of chemical equilibrium composition of aqueous systems*; Department of Civil Engineering, Massachusetts Institute of Technology, 1976.
- (19) Donat, J. R.; Lao, K. A.; Bruland, K. W. *Anal. Chim. Acta* **1994**, *284*, 547–571.
- (20) Moffett, J. W.; Brand, L. E.; Croot, P. L.; Barbeau, K. A. *Limnol. Oceanogr.* **1997**, *42*, 789–799.
- (21) Parsons, T. R.; Maita, Y.; Lalli, C. M. *A manual of chemical and biological methods for seawater analysis*; Pergamon Press: 1984.
- (22) Shoaf, W. T.; Lium, B. W. *Limnol. Oceanogr.* **1976**, *21*, 926–928.
- (23) Wright, S. W.; Thomas, D. P.; Marchant, H. J.; Higgins, H. W.; Mackey, M. D.; Mackey, D. J. *Mar. Ecol. Prog. Ser.* **1996**, *144*, 285–298.
- (24) Zapata, M.; Ayala, A. M.; Franco, J. M.; Garrido, J. L. *Chromatographia* **1987**, *23*, 26–30.
- (25) Ahner, B. A.; Price, N. M.; Morel, F. M. M. *Proc. Natl. Acad. Sci. U.S.A* **1994**, *91*, 8433–8436.
- (26) Newton, G. L.; Dorian, R.; Fahay, R. C. *Anal. Biochem.* **1981**, *114*, 383–387.
- (27) Fahey, R. C.; Newton, G. L. *Methods Enzymol.* **1987**, *143*, 85–96.
- (28) Bruland, K. W. *Limnol. Oceanogr.* **1992**, *37*, 1008–1017.
- (29) Campos, M. L. A. M.; van den Berg, C. M. G. *Anal. Chim. Acta* **1994**, *284*, 481–496.
- (30) Landing, W. M.; Bruland, K. W. *Geochim. Cosmochim. Acta* **1987**, *51*, 29–43.
- (31) Statham, P. J. *Anal. Chim. Acta* **1985**, *169*, 149–159.
- (32) Moffett, J. W.; Brand, L. E. *Limnol. Oceanogr.* **1996**, *41*, 388–395.
- (33) Davison, W.; Fones, G. R.; Harper, M.; Teasdale, P.; Zhang, H. *In-situ Monitoring of Aquatic Systems: Chemical Analysis and Speciation*; IUPAC John Wiley and Sons Ltd.: 2000.
- (34) Sunda, W. G. *Mar. Chem.* **1984**, *14*, 365–378.
- (35) Zhang, H.; Davison, W. *Anal. Chem.* **2000**, *72*, 4447–4457.
- (36) Sunda, W. G.; Huntsman, S. A. *Sci. Total Environ.* **1998**, *219*, 165–181.
- (37) O'Reilly, R.; Marshall, H. G. *Castanea* **1988**, *53*, 197–206.
- (38) Rijstenbil, J. W.; Dehairs, F.; Ehrlich, R.; Wijnholds, J. W. *Aquat. Toxicol.* **1998**, *42*, 187–209.
- (39) Vatamaniuk, O. K.; Mari, S.; Lu, Y. P.; Rea, P. A. *J. Biol. Chem.* **2000**, *275*, 31451–31459.
- (40) Hayashi, Y.; Nakagawa, C. W.; Mutoh, N.; Isobe, M.; Goto, T. *Biochem. Cell Biol.* **1991**, *66*, 288–295.

Received for review January 2, 2003. Revised manuscript received May 30, 2003. Accepted June 5, 2003.

ES0340026