Limnol. Oceanogr., 50(2), 2005, 508–515

Effects of copper, cadmium, and zinc on the production and exudation of thiols by *Emiliania huxleyi*

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Abstract

Cultures of the ubiquitous coccolithophore *Emiliania huxleyi* grown at field-relevant fixed free ion concentrations of Cu, Cd, and Zn exude a broad array of thiols, some of which increase with increasing metal ion concentration. The primary thiols released are contingent on the particular metal or combination of metals added to the culture media. Exposure to Cu results in the release of arginine-cysteine, glutamine-cysteine, and cysteine; Cd causes these thiols and glutathione to be released; and high Zn results in the synthesis and exudation of predominantly γ-glutamate-cysteine (γ-Glu-Cys). Because the free ion concentrations of Cu and Cd used in these experiments are similar to those observed in the field, active exudation could be an important source of thiols to surface seawater and thus might affect trace metal speciation in the open ocean. Intracellular thiol concentrations were also clearly affected by metal concentrations, with γ-Glu-Cys being particularly dynamic. Additionally, the shift from exponential to stationary growth in batch cultures caused approximately two- to fourfold declines in the concentrations of nearly all intracellular thiols.

There is evidence that sulfur-containing compounds of low molecular mass are an important component of the pool of ligands responsible for the nearly complete complexation of Cu in surface seawater (Tang et al. 2000; Laglera and van den Berg 2003; Ross et al. 2003), although exact chemical structures are still unknown. The nature of the ligands responsible for the complexation of Zn and Cd also remains unknown (Ellwood 2004). Field studies support the notion that the ligands for each metal are of recent biological origin (Moffett et al. 1990; Bruland et al. 1991), and culture studies have confirmed that phytoplankton have the capability of producing ligands with similar complexing strengths to those measured in the field (Moffett and Brand 1996; Leal et al. 1999; Croot et al. 2000). Specifically, Leal et al. (1999) found that high copper concentrations promoted the simultaneous release of copper-binding ligands and compounds with reduction potentials similar to those of known thiols.

We identified two novel thiols produced by *Emiliania huxleyi*, arginine-cysteine (Arg-Cys) and glutamine-cysteine (Gln-Cys), which were present at high intracellular concentrations (Dupont et al. 2004*b*). These compounds, along with cysteine, were released proportionally as copper was added to the growth media. Specific thiol–Cu(I) complexes—Cys-Cu(I), Arg-Cys-Cu(I)-Cys, and $(Cys)_2$-Cu(I)—were also identified in the culture media of copper-exposed *E. huxleyi* with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Given that the culture techniques used in that study were designed to produce high concentrations of copper ligand complexes, it remains to be determined whether the exudation of these thiols occurs at environmentally relevant concentrations of Cu. It is also important to investigate to what extent the intracellular concentrations of these novel thiols vary in response to metal exposure and other variables because this information will provide insight to the biochemical role of these compounds.

Other low–molecular mass thiols known as phytochelatins are synthesized by different algal species in response to potentially toxic elements such as Cd, Cu, Zn, and Pb (*see* review by Ahner and Morel 1999) and are also exuded by marine diatoms in response to Cd exposure (Lee et al. 1996; Wei and Ahner 2005). Although *E. huxleyi* has been observed to synthesize phytochelatins in response to metal stress (Ahner et al. 2002), the potential role of other low–molecular mass thiols such as γ-glutamate-cysteine (γ-Glu-Cys), Cys, Gln-Cys, or Arg-Cys has not been studied extensively. Because both active exudation of thiols and the release of thiols from grazing or a viral lysis event could serve as a source of metal ligands to surface seawater, determining the controls on intracellular concentrations and the exudation of low–molecular mass thiols warrants further study.

Here, we examine the effects of varying metal concentrations on the production and exudation of specific thiols by *E. huxleyi* with the use of trace metal-buffered artificial seawater (Price et al. 1988/1989) to fix free ion concentrations of Cu, Cd, and Zn at levels commonly found in natural systems. In addition, batch cultures of Cu-exposed cells were sampled at different time points to determine the effects of growth phase on intracellular thiol concentrations, which as we show, is an important variable when examining algal-produced particulate thiols.

Methods

General culture techniques—Axenic cultures of *E. huxleyi* (clone CCMP 373, Provasoli-Gulliard National Center for Culture of Marine Phytoplankton) were grown in the synthetic seawater AQUIL (Price et al. 1988/1989) with a con-

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Acknowledgments

This work was funded by an NSF grant (OCE-0002677) to B.A.A. We thank T. Goepfert for help in algal culturing and sampling and two anonymous reviewers for helpful comments and suggestions.

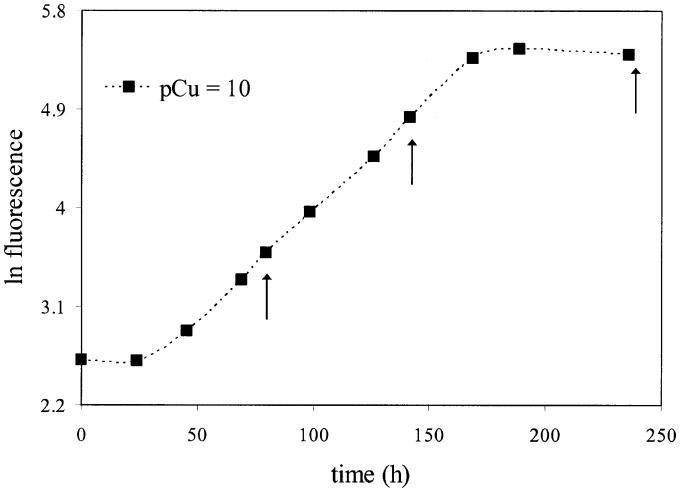

Fig. 1. Natural log of culture fluorescence versus time after culture inoculation for an *E. huxleyi* culture exposed to pCu = 10($-\log[Cu^{+2}]$ = 10). Arrows indicate timing of sampling for particulate and dissolved thiols during mid exponential, late exponential, and stationary growth (from left to right).

stant light source of 120 μmol photons m^{-2} s^{-1} at 19 ± 1°C. Batch cultures of 500 ml were grown in acid-washed polycarbonate containers, and growth was monitored by fluorescence (Turner Instruments) as a proxy for chlorophyll *a* (Chl *a*; Brand et al. 1986). Cultures were sampled during mid exponential, late exponential, and stationary growth as determined by plotting the natural log of culture fluorescence versus time following innoculation (Fig. 1).

Fixed metal ion cultures—Batch cultures were grown in AQUIL with fixed ion concentrations of Cu, Cd, or Zn controlled by an excess of ethylenediaminetetraacetic acid (EDTA), which provided a buffer against metal speciation changes potentially caused by adsorption by the cells or ligand production (Price et al. 1988/1989). Additions of stock solutions of equimolar Cu-EDTA, Cd-EDTA, or Zn-EDTA were used to increase the free ion concentrations of Cu, Cd, and Zn above the levels of the base AQUIL trace metal (Me) mixture (pCu 13.8, pZn 10.9, no cadmium). Total Me-EDTA additions required for each free ion concentration were based on equilibrium calculations made by MINEQL (Westall et al. 1976) and are summarized in Table 1. Fixed ion concentrations are reported as pMe, where pMe = $-\log[Me^{+2}]$.

Thiol measurements—Methods outlining the extraction of particulate thiols and the measurement of both dissolved and particulate thiols are described by Dupont et al. (2004*b*). To extract particulate thiols, cells were collected by gently (<50 kPa) filtering 50 ml of culture through a 25-mm Whatman GF/F filter that was subsequently stored in liquid nitrogen until processing. After removal from liquid nitrogen, the filters were immediately heated in 2 ml of 10 mmol L^{-1} methanesulfonic acid at 70°C for 2 min before homogenization with a Wheaton overhead stirrer in an ice-water bath. The slurry was then centrifuged for 10 min at 13,000 rpm and 4°C (Biofuge Fresco, Heraeus Instruments). An aliquot of supernatant (800 μl) was adjusted to pH 9 by adding 84 μl of 100 mmol L^{-1} tetraborate buffer (940 μmol L^{-1} final concentration) that also contained 10 mmol L^{-1} diethylenetriaminepentaacetic acid (DTPA, 9.3 mmol L^{-1} final concentration). An addition of 15 mmol L^{-1} dithiothreitol (DTT, 50 μmol L^{-1} final concentration) was then made 10 min before addition of the fluorescent probe monobromobimane (50 mmol L^{-1} in acetonitrile, mBBr; Molecular Probes) at a final concentration of 168 μmol L^{-1} mBBr in the sample (Ahner et al. 2002). Deviations from the published protocol of Ahner et al. (2002) include no final acidification and the exclusion of additional thiol-containing reductants after the addition of mBBr. Particulate thiol concentrations are normalized to particulate Chl *a*, which was determined by filtering 5 ml of culture onto a Whatman GF/F filter, followed by immediate extraction with 5 ml of 45 : 45 : 10 dimethyl sulfoxide (DMSO) : acetone : water (Shoaf and Lium 1976). Following incubation in the dark for 4–6 h, Chl *a* fluorescence was measured with a fluorometer (Turner Instruments) that was externally calibrated with a spectrophotometer (Beckman Instruments) and a Chl *a* solution extracted from spinach with the same DMSO : acetone : water solution. Dissolved thiol concentrations (typically 1–30 nmol L^{-1}) are also normalized to particulate Chl *a* concentrations in the media to account for differences in culture density among experiments. To measure dissolved thiols, culture media (5 ml) was gently (<50 kPa) filtered through a 25-mm Whatman GF/F filter, and duplicate aliquots (800 μl) of each filtrate were then derivatized with mBBr as described above.

HPLC thiol analysis—Monobromobimane derivatized samples were analyzed by high-performance liquid chromatography (Beckman) equipped with a Supelco Discovery reversed-phase C-16 amide column (2.1 × 250 mm) and a 100-μl injection loop. Compounds were quantified postcolumn by fluorescence detection (Gilson, excitation 310–410 nm, emission 475–650 nm). Solution A was 1% acetonitrile in 0.25% acetic acid titrated to pH 3.5 with 1 mol L^{-1}

Table 1. Cu, Cd, Zn (pZn levels are in parentheses), and EDTA added to achieve fixed ion concentrations of Cu, Cd, and Zn in AQUIL. Total metal concentrations are in mol L^{-1}.

	pMe				
Metal	13.8	12 (9)	11 (8)	10 (7)	Total EDTA
Cu	1.96×10^{-9}	1.2×10^{-7}	1.2×10^{-6}	1.2×10^{-5}	10 μmol L^{-1}+Cu_T
Cd	—	3×10^{-8}	3×10^{-7}	3×10^{-6}	10 μmol L^{-1}+Cd_T
Zn	—	6×10^{-6}	6×10^{-5}	6×10^{-4}	10 μmol L^{-1}+Zn_T

Table 2. Particulate Chl *a* concentrations measured at three growth stages (columns 2–4), and growth rates (column 5) of *E. huxleyi* calculated for the exponential phase under different trace metal regimes. Growth phase was determined by examining ln(culture fluorescence) versus time plots (Fig. 1).*

Free metal concentration	Chl *a* (ng ml^{-1}) Mid exponential	Late exponential	Stationary	μ (d^{-1})
pCu 13.8	42.4	96.9	121.9	0.39
12	36.6	88.8	124.0	0.39
11	42.0	103.3	131.8	0.41
10	26.1	85.8	93.7	0.39
pCd 12	—	67.4	—	0.50
11	14.6	—	—	0.32
10	12.5	—	—	0.40
pZn 9	34.0	—	—	0.33
8	55.7	—	—	0.37
7	7.6	—	—	0.13
pCu 11, pZn 9, pCd 11	31.5	—	—	0.50
pCu 11, pZn 9, pCd 10	33.4	—	—	0.45

* —, the cultures were not sampled during these phases of the growth curve.

NH_4OH, and solution B was pure acetonitrile. The elution gradient was as follows: 0% B for 8 min, a linear increase to 30% B over 60 min, a linear increase to 60% B in 2 min to flush the column, a linear decrease to 0% B over 10 min, and a final 20 min at 0% B to re-equilibrate the column. The flow rate was 150 μl min^{-1}. Standards of Arg-Cys, Gln-Cys (Cell Essentials), Cys, γ-Glu-Cys, and reduced glutathione (GSH; Sigma) were analyzed to verify retention times and develop standard curves for peak area calibration. For GSH, Cys, and other singly labeled thiols, the sensitivity of the method is 100 fmol per injection (1 nmol L^{-1}). Low concentrations (<5 nmol L^{-1}) of phytochelatins ($n = 2$) are not quantifiable by this method because of coelution with a small reagent peak and were not analyzed in this study.

Results

Fixed free copper experiments—The concentrations of dissolved thiols were measured in the growth media during mid exponential growth in duplicate cultures of *E. huxleyi* grown at a range of [Cu^{+2}]. The growth rates of the cultures were not significantly affected by the concentrations of copper used (Table 2), a result previously observed with *E. huxleyi* strain Hay and Mohler (Brand et al. 1986). In general, dissolved thiol concentrations increased with increasing [Cu^{+2}] (Fig. 2). At all copper levels, Gln-Cys concentrations in the culture media were highest of the thiols measured, followed by Cys and Arg-Cys (Fig. 2A,B,C). GSH was present only in trace amounts at low Cu concentrations and increased slightly with increasing Cu (Fig. 2D). Because of a coelution with a small reagent peak, low nanomolar concentrations of γ-Glu-Cys could not be measured in this ex-

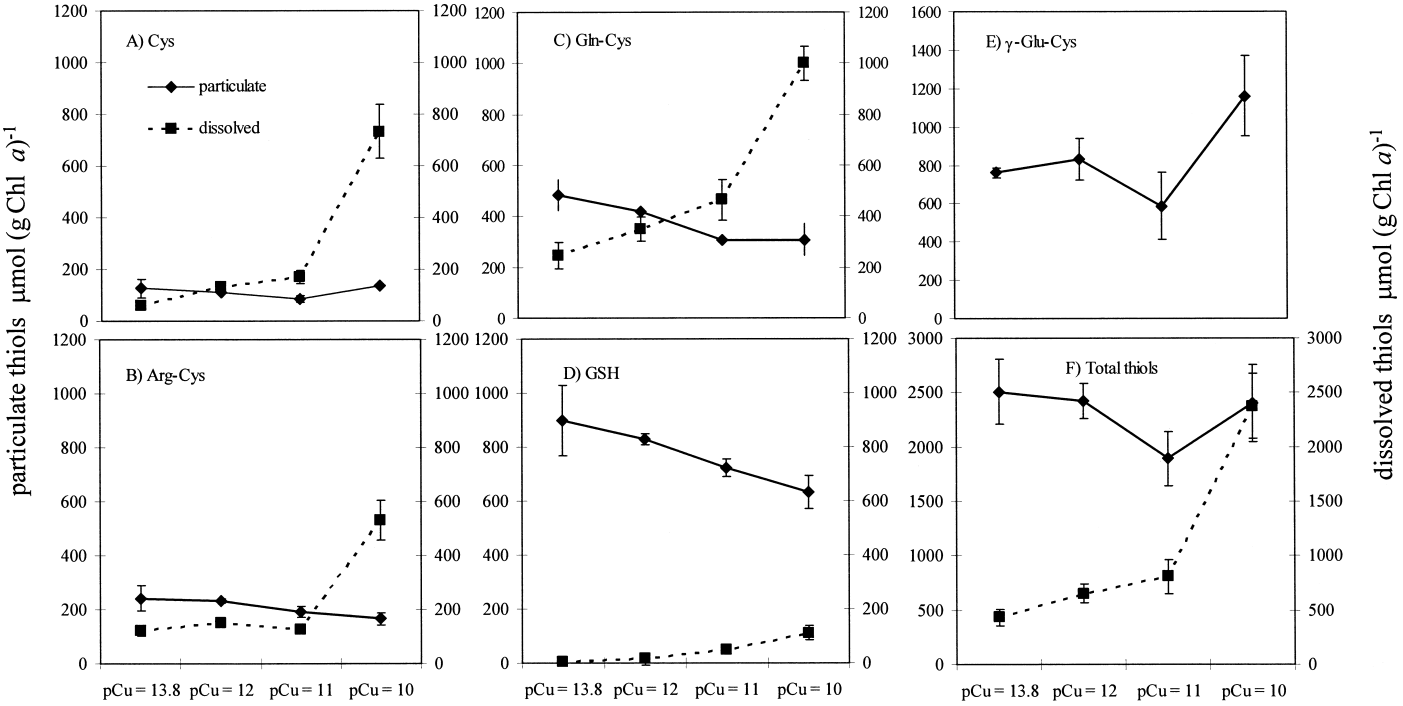

Fig. 2. Particulate (left axis) and dissolved (right axis) (A) Cys, (B) Arg-Cys, (C) Gln-Cys, (D) GSH, (E) γ-Glu-Cys, and (F) the sum of these five thiols from mid exponential growth *E. huxleyi* cultures exposed to a range of [Cu^{+2}]. Both particulate and dissolved thiol concentrations were normalized to particulate Chl *a* (*see text for details*). Absolute dissolved concentrations can be calculated from the Chl *a* concentrations given in Table 1; for example, the concentration of Gln-Cys measured at pCu = 10 was 26 nmol L^{-1} (1,000 μmol Gln-Cys g^{-1} Chl *a* times 26 ng Chl *a* ml^{-1}). Error bars are the range of measurements from duplicate cultures.

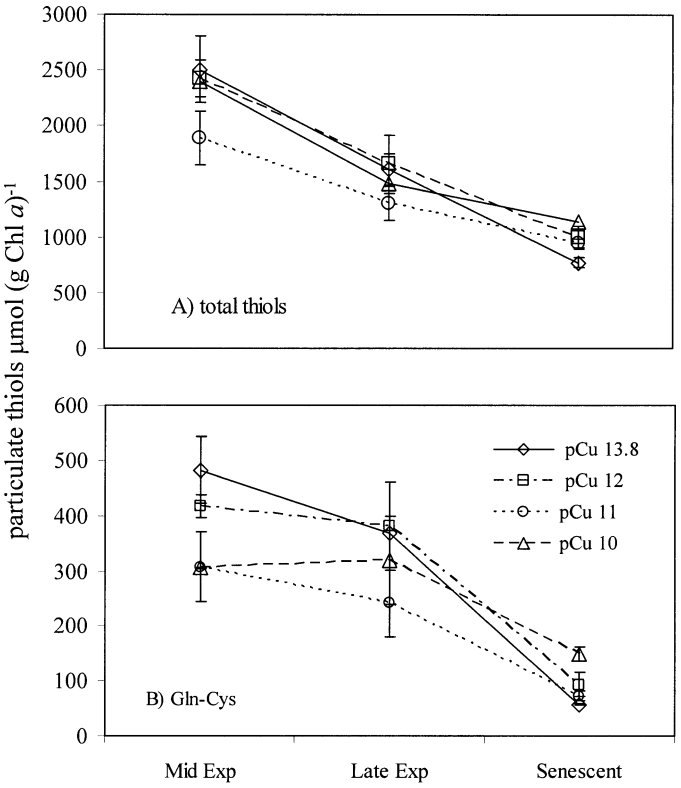

Fig. 3. (A) Sum of particulate Cys, Arg-Cys, Gln-Cys, GSH, and γ-Glu-Cys concentrations and (B) Gln-Cys concentrations (µmol g^{-1} Chl *a*) from *E. huxleyi* cultures grown at a range of [Cu^{+2}] and sampled at three different stages of growth as shown in Fig. 1. Error bars are the range of measurements from duplicate cultures.

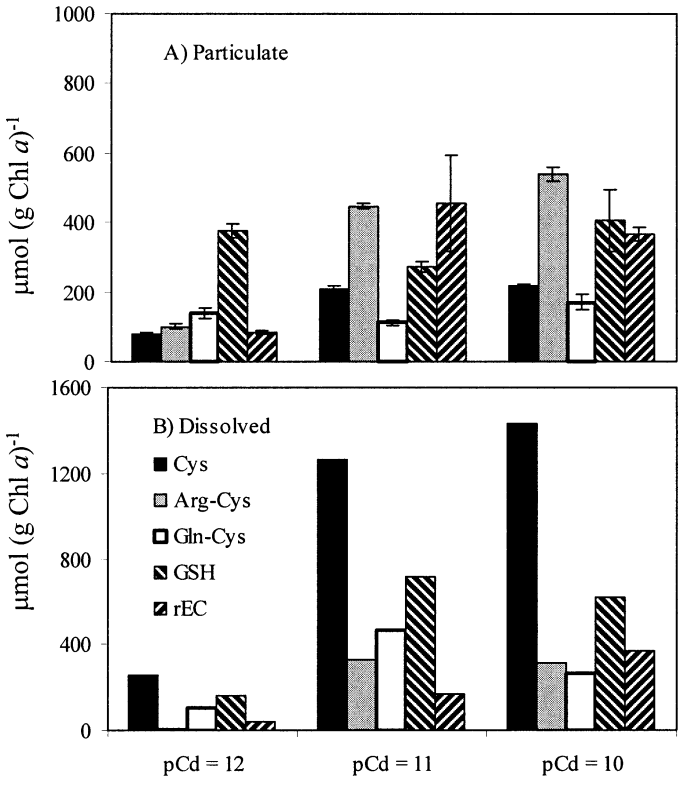

Fig. 4. (A) Particulate thiols from exponential growth *E. huxleyi* cultures exposed to a range of [Cd^{+2}]. Error bars are the range of duplicate measurements from the same culture. (B) Dissolved thiol concentrations from the same cultures. Duplicate measurements of dissolved thiols were not taken because the error from sample treatment for dissolved thiols is <5%. rEC denotes γ-Glu-Cys.

periment, although intracellular concentrations were high enough to be quantifiable.

Particulate thiols were measured during mid exponential growth to determine the effects of a range of [Cu^{+2}] on the concentrations of intracellular thiols. Intracellular concentrations of GSH and Gln-Cys declined with increasing copper concentrations (Fig. 2C,D), whereas γ-Glu-Cys concentrations were highest in the pCu 10 cultures (Fig. 2E), as was also seen in a previous study (Ahner et al. 2002). Cys and Arg-Cys concentrations were unaffected by increasing [Cu^{+2}] (Fig. 2A,B). Given the high intracellular concentrations of GSH and the low levels observed in the growth media (Fig. 2D), it is unlikely that cell leakage or breakage during filtration contributed significantly to the measured dissolved thiols.

These cultures were sampled near the end of exponential growth and again early in stationary phase to determine the effects of growth phase on thiol production. The shift from exponential to stationary growth is accompanied by a two- to fourfold decline in the sum of the concentrations of the measured thiols, with less of a decline at higher Cu concentrations (Fig. 3A). Arg-Cys, Cys, GSH, and γ-Glu-Cys concentrations each declined approximately twofold (slightly less at higher copper concentrations; data not shown), whereas Gln-Cys concentrations fell more than eightfold at pCu 13.8, approximately fourfold at pCu 12 and pCu 11, and only twofold at pCu 10 (Fig. 3B).

Fixed free cadmium experiments—Particulate and dissolved thiols were measured in cultures grown at pCd 12, pCd 11, and pCd 10 during exponential growth. The growth rates of the pCd 11 and pCd 10 cultures were 36% and 20% lower, respectively, than the growth rate of the pCd 12 culture (Table 2). Thiol exudation is prompted by increased [Cd^{+2}]; higher concentrations of all five thiols were observed in the culture media with the increase from pCd 12 to pCd 11, whereas only Cys and γ-Glu-Cys concentrations increased from pCd 11 to pCd 10 (Fig. 4B). Cys was the primary exudate, although appreciable amounts of the other thiols, particularly GSH, were also observed (Fig. 4B).

As [Cd^{+2}] increased from pCd 12 to pCd 11, elevated intracellular concentrations of Cys, Arg-Cys, and γ-Glu-Cys were observed, whereas Gln-Cys and GSH concentrations were more stable (Fig. 4A). Because the cultures were sampled at the same time and the growth rates were depressed at higher Cd (Table 2), the observed differences in intracellular thiols between cadmium cultures might in part be the result of a growth phase closer to senescence in the faster growing pCd 12 culture. However, as shown in the copper experiments, the shift from late exponential to stationary growth causes approximately twofold declines in thiol con-

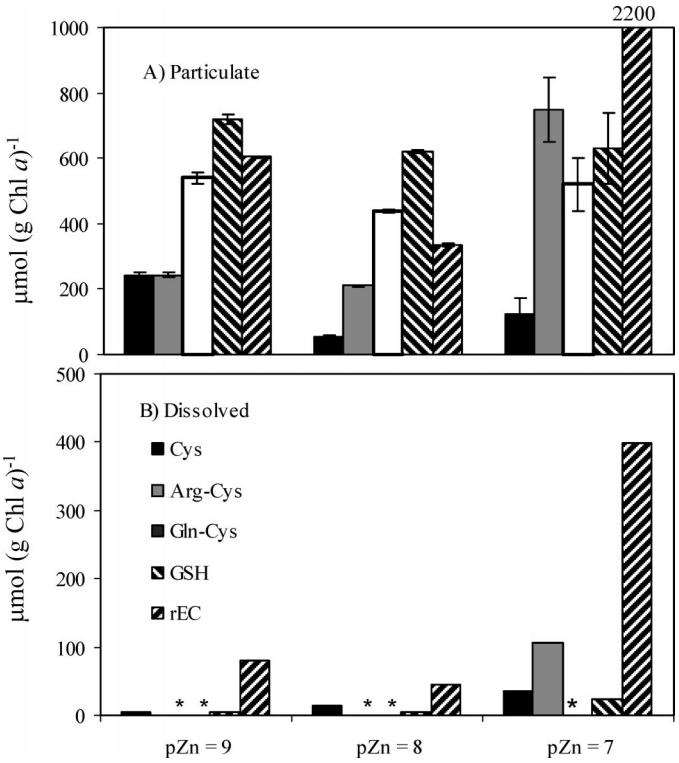

Fig. 5. (A) Particulate thiols from exponential growth *E. huxleyi* cultures exposed to a range of [Zn^{+2}]. Error bars are the range of duplicate measurements from the same culture. The γ-Glu-Cys concentration in the pZn 7 culture was ~2,200 µmol g^{-1} Chl *a*. (B) Dissolved thiol concentrations from the same cultures. rEC denotes γ-Glu-Cys. * Concentration was below detection.

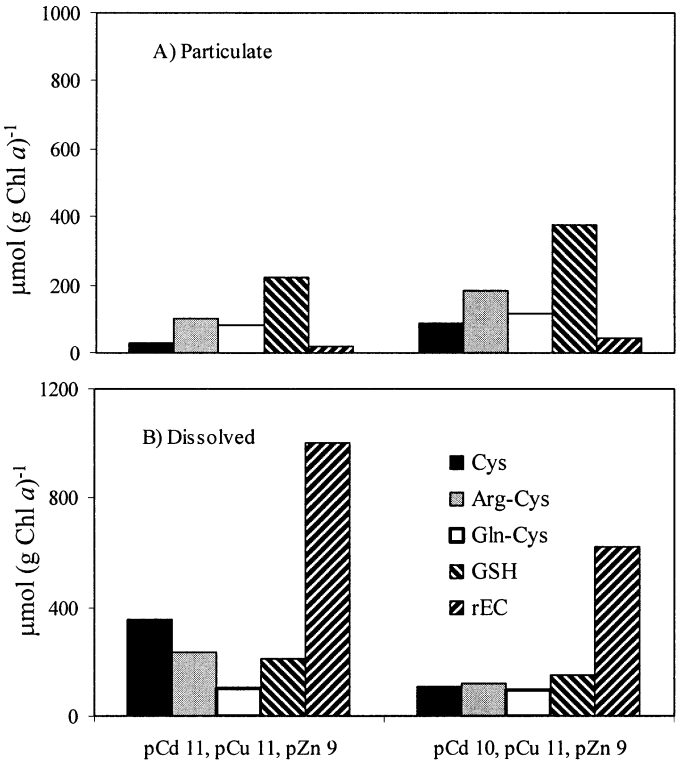

Fig. 6. (A) Particulate thiols from exponential growth *E. huxleyi* cultures grown at pCu 11 and pZn 9 with Cd at either pCd 11 or pCd 10. (B) Dissolved thiol concentrations from the same cultures. rEC denotes γ-Glu-Cys.

centrations (Fig. 3A), whereas the increases observed with increasing Cd are two- to fourfold and therefore at least partially attributable to a Cd-induced change. On the other hand, the observed γ-Glu-Cys changes could be completely explained by the differences in culture growth phase.

Fixed free zinc experiments—Cultures of *E. huxleyi* grown at pZn 9, pZn 8, and pZn 7 were sampled for dissolved thiols during exponential growth. At pZn 7, the growth rate was only a third of that of the pZn 9 and pZn 8 cultures (Table 2). Compared with the exudation observed in the control and elevated Cu and Cd experiments, the response to Zn is very distinct, and overall dissolved thiol concentrations were much lower (Fig. 5B; note differences in scale between Figs. 2, 4B, 5B). Proportionally much more γ-Glu-Cys was released into the growth media along with smaller amounts of Cys and GSH (Fig. 5B). Gln-Cys and Arg-Cys remained below detection, except at pZn 7, for which Arg-Cys was present in concentrations higher than those of Cys or GSH (Fig. 5B). The amounts released were highest in the pZn 7 culture, and there was little difference between the pZn 9 and pZn 8 cultures (Fig. 5B).

Particulate thiols were measured during exponential growth. Increasing pZn concentrations from 9 to 7 elicited changes that were distinct from those caused by Cd or Cu. Cys, Gln-Cys, and GSH concentrations did not vary significantly in response to [Zn^{+2}], whereas elevated Arg-Cys and γ-Glu-Cys levels were seen at the highest [Zn^{+2}] (Fig. 5A). Again, as with Cd, part of the difference between the pZn 7 culture and the others could be because of differences in cell density, but the magnitude of the changes (greater than threefold increases in both γ-Glu-Cys and Arg-Cys) is more than can be explained in this manner.

Incubations with mixes of Cd, Cu, and Zn—To determine whether competitive metal interactions are important with respect to low–molecular mass thiol production and exudation in *E. huxleyi*, we grew cultures with simultaneous additions of pZn 9, pCu 11, and either pCd 11 or pCd 10. These cultures were only sampled during exponential growth. Both cultures with elevated [Zn^{+2}], [Cu^{+2}], and [Cd^{+2}] had higher growth rates than the corresponding cadmium culture (Table 2), which would suggest that the high Cu and Zn concentrations protected the cells from cadmium toxicity. As previously described, high Cd causes increases in intracellular Arg-Cys and Cys, but in this experiment, these thiols were not elevated relative to other thiols (Fig. 6A). Indeed, the relative ratios or pattern of intracellular thiols resembles that of the pCd 12 culture, although total concentrations were much lower (Figs. 4A, 6A). Additionally, γ-Glu-Cys concentrations were the lowest observed in any experiment (Fig. 6A). Because both cultures were still in exponential growth and the culture densities were only marginally higher than those in the Cd-only experiment (and nearly the same as the Cu-only, Table 2), growth phase and

potential nutrient depletion cannot be used to explain the low intracellular thiol concentrations.

In the presence of high Cu and Zn, the amount and composition of thiols released into the growth media were nearly the same at pCd 11 and pCd 10 (Fig. 6B). The primary exudate was γ-Glu-Cys, with the other thiols being present in much lower amounts (Fig. 6B); this is in contrast to those cultures with only elevated cadmium, in which Cys and GSH were the primary exudates (Fig. 4B). The pattern of exuded thiols resembles that observed in the Zn experiment (Fig. 5B) and is very different from the relative ratios seen in the Cu or Cd experiments (Figs. 2, 4B).

Discussion

Effects of metals on thiol production and exudation—Particulate thiol concentrations in *E. huxleyi* are dependent on free metal concentrations of the culture media. In addition, the concentrations of all of the low–molecular mass thiols examined here decline as the cultures progress from exponential to stationary growth, but the extent to which they decrease is modulated by metals. Although nutrient concentrations were not measured, it is possible that nutrient depletion caused the observed decreases in particulate thiol concentrations with the shift from exponential to stationary growth. Cultures of *Thalassiosira pseudonana* grown under nitrogen-limiting conditions have lower ambient levels of glutathione and phytochelatins (Rijstenbil et al. 1998), and nutrient limitation has been suggested to constrain the synthesis of phytochelatin in response to metal stress in some marine algae (Ahner et al. 2002). Sulfate assimilation can be curtailed by nitrogen limitation in higher plants (Koprivova et al. 2000), which might explain the effects observed here. As the cells approach nutrient limitation, the synthesis of nonessential sulfur-containing compounds could be downregulated, and because higher thiol concentrations might be required when a metal stress is imposed, less of a decrease is observed in the cultures exposed to higher metal concentrations.

Thiols are exuded by *E. huxleyi*, even in the control media, and amounts exuded appear to increase with increasing metal concentrations, whereas the relative amounts of individual thiols varies according to the metal added. We contend that the measured dissolved thiols are selectively and actively exuded by the algae as opposed to leaking from cells during filtration because the primary thiols observed are in most cases different from the most abundant intracellular thiols. Furthermore, in the cases where high levels of a specific thiol were measured in solution, depressed intracellular levels were concurrently observed (e.g., Gln-Cys; Fig. 2C). Increasing thiol exudation with increased $[Me^{+2}]$ has also been observed in the coastal diatoms *T. pseudonana* (phytochelatin, $n = 2$; Wei and Ahner in press) and *T. weissflogii* (GSH, γ-Glu-Cys, and phytochelatin, $n = 2$; C. L. Dupont unpubl. data). Although it is likely that nutrient or growth status will also have an effect on thiol exudation rates, the use of batch cultures in these experiments in which dissolved thiols accumulate over time obscures these potential effects.

Biological role of thiols—The production and exudation of various thiols in response to increasing metal stress implies a complex biochemical response by *E. huxleyi*. Here, we showed that intracellular γ-Glu-Cys increases with increasing Cu, Cd, or Zn concentrations, a phenomenon that has been noted before for Cd and Cu in *E. huxleyi* and some other algae (Ahner et al. 2002). Although GSH is usually the primary thiol in particulate samples collected from coastal seawater (Wei et al. 2003; Dupont et al. 2004*a*), concentrations of particulate γ-Glu-Cys were higher than particulate GSH concentrations in a contaminated estuary (Tang et al. 2000). The intracellular response, along with its exudation in response to Zn, provides strong evidence that this thiol plays a heretofore unrecognized role in metal detoxification. γ-Glu-Cys might directly buffer increasing intracellular metal concentrations through sulfhydryl coordination, like the structurally similar glutathione (Rabenstein 1989); perhaps in the case of Zn, these intracellular complexes are then exuded, like phytochelatin–Cd complexes (Lee et al. 1996). Phytochelatins, which have been well studied in marine algae, can be synthesized from γ-Glu-Cys in fission yeast (Hayashi et al. 1991); conceivably, this is the case in *E. huxleyi* and some other algae and might also explain the intracellular increases in response to Cu, Cd, and Zn.

Like γ-Glu-Cys, Cys and Arg-Cys appear to play a previously unrecognized role in generalized metal detoxification in *E. huxleyi*. Both thiols are released in response to high concentrations of copper (Dupont et al. 2004*b*). Here, we show that Cys is exuded by *E. huxleyi* in response to environmentally relevant concentrations of Cu. Additionally, Cys is the primary thiol released in response to Cd and a minor exudate in response to Zn. Arg-Cys was a minor component of the thiols exuded at the higher concentrations of Cd and Zn, and like γ-Glu-Cys, elevated Cd or Zn can stimulate high intracellular concentrations of Arg-Cys.

In general, intracellular glutathione does not vary in response to Cu or Cd additions, although Cu-induced depressions have been observed (Ahner et al. 2002; Pinto et al. 2003; Fig. 2D) and appreciable amounts are exuded in response to Cd. Because glutathione has multiple metabolic roles in addition to metal homeostasis (Meister and Anderson 1983), it is perhaps not surprising that intracellular GSH concentrations are stable with respect to metal concentrations. Light-induced, as opposed to metal-induced, oxidative stress could be a more important control over intracellular GSH concentrations in marine algae because we have shown twofold variations in both laboratory cultures and natural assemblages during a diurnal light cycle (Dupont et al. 2004*a*).

Although it is exuded into the extracellular milieu in response to copper (Dupont et al. 2004*b*; this study), intracellular Gln-Cys concentrations are stable with increasing metal concentrations. However, the transition from mid exponential to senescent phase in batch cultures is accompanied by large decreases in Gln-Cys concentrations (Fig. 3B). Declining concentrations of nitrate and phosphate in the growth media might explain this trend. We have previously shown that in nitrogen-starved *E. huxleyi* cells, Gln-Cys concentrations increase over fivefold 24 h after the addition of nitrate,

Table 3. Free ion concentrations ($-\log[Me^{2+}]$ of Zn, Co, Mn, Cu, and Cd) in the open ocean and in AQUIL used during the experiments with elevated Cu, Cd, and Zn (Fig. 6).

	Free ion concentration			
Metal	AQUIL*	Open ocean	Location	Reference
$[Zn^{+2}]$	9	12.7	North Pacific	Bruland 1989
$[Co^{+2}]$	10.88	17–14	Sargasso Sea	Saito and Moffett 2001
$[Mn^{+2}]$	8.27	9.8–9.3	North Pacific	Bruland and Franks 1983
$[Cu^{+2}]$	11	12.3–11.2	Sargasso Sea	Mann et al. 2002
$[Cd^{+2}]$	11–10	13.7–12	Equatorial Pacific	Bruland 1992

* Price et al. (1988/1989) or MINEQL (see ''Methods'').

and we have hypothesized that this compound could play a role in nitrogen assimilation (Dupont et al. 2004*b*).

Combinatorial effects—Although limited in scope, the cultures grown at a mixture of elevated Cu, Zn, and Cd indicate a distinct biochemical response to a mixture of metals that cannot be explained by simple additive effects or even competitive metal uptake. High $[Zn^{+2}]$ and $[Mn^{+2}]$ have been shown to protect diatoms from Cd toxicity by decreasing Cd uptake rates (Sunda and Huntsman 1998), and the intracellular concentrations of phytochelatins in laboratory cultures and natural algal assemblages amended simultaneously with Cd, Cu, and Zn are lower than those in parallel incubations that have been augmented with only Cd, Cu, or Zn (Wei et al. 2003). Simultaneous additions of Pb and Cd to *E. huxleyi* cultures resulted in lower ligand exudation and copper uptake than would be expected through a simple additive effect (Vasconcelos and Leal 2001). Here, we found that the growth rates of *E. huxleyi* simultaneously exposed to high Cu, Cd, and Zn were similar to those of the control cultures (pCu 13.8), whereas the biochemical response, as demonstrated by the profile of thiol production and exudation, was quite unique. The dissimilarities in the thiol production and exudation profiles between the mixed metal cultures and the low-metal control cultures (pCu 13.8) indicates that the protective mechanism mediated by high levels of several metals might be more involved than the simple suppression of uptake from competitive interactions at the membrane transport sites.

However, in the open ocean, where *E. huxleyi* thrives, the free ion concentrations of Mn, Zn, and Co are much lower than in our growth media (Table 3), and uptake rates of Cu and Cd are greater under conditions of low Mn, Zn, and Co (Sunda and Huntsman 1998). The Cu and Cd concentrations in surface seawater approach the lower concentrations used in this study (Table 3); therefore, uptake rates of Cu and Cd and the resulting thiol production and exudation might be greater in the field than we measured in our culture studies at corresponding metal concentrations. Supporting this speculation, surprisingly high particulate phytochelatin concentrations were observed in an oligotrophic region of the equatorial Pacific, presumably because of the synergistic effect on toxic metal uptake caused by the low concentrations of nutrient metals (Fe, Co) in this region (Ahner et al. 1998).

The culture studies reported here show that thiol exudation is modulated by metal availability and might explain nanomolar concentrations of dissolved thiols reported in the open ocean (Le Gall and van den Berg 1998). The exudation of low–molecular mass thiols can affect copper chemistry through the formation of thiol-Cu(I) complexes (Leal and van den Berg 1998; Dupont et al. 2004*b*). Thiols can also bind strongly to Zn, Pb, Cd, and Hg (Rabenstein 1989), and all of these metals have been shown to be complexed by unidentified organic ligands in surface seawater (Capodaglio et al. 1990; Mason et al. 1998; Ellwood 2004). Even low nanomolar concentrations of biogenic thiols will influence the speciation of Cu, Cd, Zn, Pb, and Hg in the absence of stronger chelators; thus, even low levels of thiol exudation by marine phytoplankton could alter metal chemistry in the seawater.

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Received: 2 August 2004
Accepted: 4 November 2004
Amended: 16 November 2004