Limnol. Oceanogr., 49(5), 2004, 1754–1762

Novel copper-binding and nitrogen-rich thiols produced and exuded by *Emiliania huxleyi*

Christopher L. Dupont[1]
Biological and Environmental Engineering, Cornell University, Ithaca, New York 14853

Robert K. Nelson
Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Massachusetts 02543

Saj Bashir[2]
Plant Science, Cornell University, Ithaca, New York 14853

James W. Moffett
Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Massachusetts 02543

Beth A. Ahner
Biological and Environmental Engineering, Cornell University, Ithaca, New York 14853

Abstract

In surface seawater, dissolved copper (Cu) is predominantly bound by a small pool of strong ligands, with further complexation by a larger pool of weaker ligands. Although the chemical structure of both classes of ligands observed in the open ocean remains unknown, it is believed that the stronger ligands are of biological origin, and it has been hypothesized that they are thiols. Using precolumn derivatization high-performance liquid chromatography electrospray ionization ion-trap mass spectrometry, we found that the ubiquitous coccolithophorid *Emiliania huxleyi* constitutively produces two previously uncharacterized thiols, arginine–cysteine and glutamine–cysteine, in high intracellular concentrations. *E. huxleyi* exudes these novel thiols, along with cysteine, in response to increased Cu concentrations in the growth media. Furthermore, stable Cu (I) complexes with the exuded thiols were observed in the growth media using matrix-assisted laser desorption time-of-flight mass spectrometry following size exclusion chromatography. Additionally, *E. huxleyi* appears to utilize these novel thiols in nitrogen storage and assimilation, as they are rapidly synthesized upon nitrogen addition to a nitrogen-depleted culture. The identification of specific ligands produced by this organism will facilitate further linkages between this likely source and the pools of Cu-binding ligands observed in surface seawater, while the discovery of novel thiols signifies the presence of unique and previously unknown biochemical pathways in *E. huxleyi.*

In surface seawater, copper is complexed by a pool of organic ligands that decreases the bioavailable free ion concentrations from toxic to nutritive levels (van den Berg 1984; Coale and Bruland 1988; Moffett 1995), and variations in copper (Cu) complexation can influence community composition in marine systems (Moffett et al. 1997; Mann et al. 2002). There is evidence from the field that these ligands are of recent biological origin (Coale and Bruland 1988; Moffett 1991), and culture studies have revealed that ligands can be produced by phytoplankton, including *Emiliania huxleyi*, specifically to chelate metals such as copper (Moffett and Brand 1996; Leal et al. 1999; Croot et al. 2000). While the chemical structures of the ligands in the field and culture studies remain unknown, there is increasing evidence that sulfur-containing compounds of low molecular weight are an important component of the ligand pool (Leal et al. 1999; Ross et al. 2003; Vachet et al. 2003).

Thiols are organic compounds containing a sulfhydryl moiety, a functional group that readily binds many trace metals. Specialized thiol-containing compounds called phytochelatins (PCs) are synthesized enzymatically from glutathione (GSH) by marine algae to chelate cadmium (Cd) and other metals, such as Cu, zinc (Zn), and lead (Pb) (Ahner and Morel 1999). It is unknown to what extent other intracellular thiols, such as cysteine (Cys) and GSH, participate in the detoxification of trace metals. In some phytoplankton, and in *E. huxleyi* in particular, intracellular concentrations of γ-Glutamate-Cysteine (γ−Glu-Cys) and Cys, both biochemical precursors to GSH (and hence precursors to PCs), increase in response to Cd and Cu (Ahner et al. 2002), indicating that they play a role in metal detoxification.

Thiols may also affect trace metal chemistry in the extracellular environment. The marine diatom *Thalassiosira weissflogii* has been shown to exude phytochelatin concurrently with Cd (Lee et al. 1996); this exudation is believed to be an important component of metal detoxification for

[1] To whom correspondence should be addressed. Present address: Scripps Institution of Oceanography, University of California San Diego, La Jolla, California 92093 (cdupont@ucsd.edu).

[2] Present address: Department of Chemistry, Texas A&M University at Kingsville, Kingsville, Texas 78363.

marine phytoplankton in general, but it has not been studied in organisms other than diatoms. *E. huxleyi* have been shown to exude compounds with voltammetric characteristics similar to thiols in response to Cu (Leal et al. 1999), but the identity of the actual compound or set of compounds, though hypothesized to be glutathione, was not determined. In addition, the concentrations of Cu-binding ligands and those of the putative thiols in the growth media were shown to increase concurrently (Leal et al. 1999), yet it has not been shown that biologically exuded thiols actually bind Cu in culture media. In this system, it is also unknown if intracellular thiols are released indiscriminately or if specific compounds (as appears to be the case with PCs and Cd in diatoms) are exuded.

The exudation of copper-binding ligands by *E. huxleyi* could have dramatic effects upon copper speciation in large parts of the open ocean. The most abundant coccolithophore on earth, *E. huxleyi* forms large blooms in midlatitude regions that may also affect planetary albedo (Tyrrell et al. 1999), carbon export (Fernandez et al. 1993), and the oceanic flux of cloud-inducing dimethyl sulfide to the atmosphere (Andreae and Crutzen 1997). Recently, blooms have extended into more polar waters, with persistent blooms being observed in the Bering Sea (Napp and Hunt 2001), and the reasons for this biogeographical shift are not clear. Known to have a high copper tolerance (Brand et al. 1986), the way that this organism handles toxic trace metals may partially explain its ecological success.

The goal of this work was to identify the thiols produced and exuded by *E. huxleyi* in response to copper and to determine if they do affect copper speciation in the extracellular environment. High-performance liquid chromatography was coupled with electrospray ionization ion-trap mass spectrometry (HPLC ESI-ITMS) to identify both known and previously unknown thiol-containing compounds. Size-exclusion chromatography and matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) were used to identify specific thiol-Cu complexes. Further information regarding the effect of this organism on copper speciation in the experimental medium was obtained using competing ligand exchange adsorptive cathodic stripping voltammetry (CLE-ACSV) with the competing ligand benzoylacetone (bzac).

Materials and methods

General culture techniques—Axenic cultures of *E. huxleyi* (clone CCMP 373, Provasoli-Guillard National Center for Culture of Marine Phytoplankton) were grown in the synthetic seawater AQUIL (Price et al. 1988/1989) with a constant light source of 120 μmol photons m^{-2} s^{-1} at 19 + 1°C. Batch cultures were grown in acid-washed polycarbonate containers and growth was monitored noninvasively using fluorescence (Turner Instruments) as a proxy for chlorophyll *a* (Chl *a*) (Brand et al. 1986). Exponentially growing cultures were gently filtered onto polycarbonate filters that were previously washed with 1 M HCl and then with Milli-Q water. The cells were rinsed with trace-metal–free AQUIL (produced by passage over a Chelex-100 resin) and resuspended to a density of 200,000 cells ml^{-1} in Chelex-purified AQUIL with no added trace metals or EDTA. For one culture, additions of 50 nmol L^{-1} copper sulfate were made daily to the resuspended cultures. For the other culture, no copper additions were made. Duplicate samples for dissolved thiols, particulate thiols, particulate Chl *a*, cell counts, and dissolved copper were taken from both cultures 24 hr after each copper addition to the copper-treated culture. Following fixation with Lugol's solution, the algal cells were counted using a Levi hemocytometer (Sournia 1978). Cultures were checked for axenicity during cell counts and by plating the stock culture on both normal and seawater agar plates.

Nitrate- or phosphate-limited cultures—AQUIL was prepared as directed in (Price et al. 1988/1989), except without added nitrate or phosphate. Chelex-purified and filter-sterilized sodium nitrate and sodium phosphate were added prior to culture inoculation. Axenic cultures of *E. huxleyi* were grown with only 5 μmol L^{-1} of added nitrate and 10 μmol L^{-1} phosphate to create nitrogen-limiting conditions (N to P ratio, 1 : 2). The cultures were allowed to grow until senescence (when culture fluorescence and cell numbers no longer increased exponentially), at which point 100 μmol L^{-1} sodium nitrate was added. Particulate thiols were sampled for the next 48 hr. Separate cultures were also grown with 1 μmol L^{-1} phosphate and 50 μmol L^{-1} nitrate to generate phosphate-limiting conditions (N to P ratio, 50 : 1). An addition of 10 μmol L^{-1} sodium phosphate was made to the resulting senescent cultures, from which samples for particulate thiols were taken for the next 48 hr.

Particulate thiols—Cells were collected by gently (<7.25 Pa) filtering 50 ml of culture through a 25-mm Whatman GF/F filter that was subsequently stored in liquid nitrogen until processing. Upon 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 at 4°C (Biofuge Fresco, Heraeus Instruments). An aliquot of supernatant (800 μl) was adjusted to a pH of 9 by adding 84 μl of 100 mmol L^{-1} borate buffer (9.4 mmol L^{-1} final concentration) that also contained 10 mmol L^{-1} diethylenetriaminepentaacetic acid (DTPA, 940 μmol L^{-1} final concentration). An addition of 15 mmol L^{-1} dithiothreitol (DTT, 50 μmol L^{-1} final concentration) was then made 10 min prior to the 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 following 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 hr, Chl *a* fluorescence was measured with a fluorometer (Turner Instruments) that was externally calibrated with a spectropho-

tometer (Beckman Instruments) and Chl *a* solution extracted from spinach using the same DMSO : acetone : water solution.

Dissolved thiols—Culture media (5 ml) was gently (<7.25 Pa) 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. For some samples, a separate aliquot was titrated to pH 3 or pH 1 using 1 mol L^{-1} HCl and allowed to equilibrate for 1 hr. At this point, DTPA was added, and the pH was brought back to 9 with 100 mmol L^{-1} borate buffer prior to DTT and mBBr additions.

HPLC thiol analysis—Monobromobimane derivatized samples were analyzed with a Beckman HPLC equipped with a Supelco Discovery reverse-phase C-16 amide column (2.1 × 250 mm) and a 100-μl injection loop. Compounds were quantified postcolumn using 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} NH_4OH, while 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 in order 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 GSH (Sigma) were analyzed in order to verify retention times and to 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 are not measurable by this method because of coelution with a small reagent peak and were not analyzed in this study.

Ion trap mass spectrometry—Thiol compounds isolated from *E. huxleyi* cell extracts were derivatized with mBBr and analyzed using HPLC coupled to an ion-trap mass spectrometer using electrospray ionization (ESI-ITMS). The ESI-ITMS was run in positive ion mode (Thermo-Finnegan Mat LCQ, MS Scan range = 100–1,000 amu; spray voltage: 3.5 kV; spray current: 4.8 mA; capillary voltage: 16.0 V; capillary temperature: 135°C). The HPLC conditions were the same as described above, with the exception of solution A, which was 1% acetonitrile in 0.025% acetic acid titrated to a pH of 3.5 with 1 mol L^{-1} NH_4OH. The molecular ions were isolated in the ion trap and then sequentially fragmented, using helium (He) as the collision gas. Identification of the unknown mBBr labeled thiol compounds was accomplished by comparing the HPLC retention times and ESI-ITMS fragmentation patterns of the unknown compounds with the synthetic dipeptides Arg-Cys and Gln-Cys (Cell Essentials) and with Cys, γ-Glu-Cys, and GSH (Sigma).

Identification of thiol-Cu complexes—Copper-exposed *E. huxleyi* cultures were filtered through polycarbonate filters (Millipore 0.2 μm) that had been washed with 1 mol L^{-1} HCl and Milli-Q water. The resulting filtrate was fractionated on a size exclusion column (Alltech Macrosphere GPC, 250 × 4.6 mm; Buffer: 10 mmol L^{-1} : 1 mmol L^{-1} NaCl : Tris, pH 8.1; flow rate: 0.3 ml min^{-1}) with an HPLC equipped with a 200-μl injection loop. Fractions were collected and thiol and copper concentrations in the fractions were determined using HPLC and graphite furnace atomic absorption spectroscopy (GFAAS), as described below. The copper- and thiol-containing fractions from multiple HPLC runs were pooled and lyophilized. The lyophilized samples were analyzed using MALDI-TOF MS (Bruker Reflex IV) run in positive ion mode with sinaptic acid as a matrix. To verify peak identities, observed MS profiles were compared with theoretical isotope ratio profiles. This was repeated with the medium from a non–copper-exposed *E. huxleyi* culture as a control. Sodium, potassium, and matrix peaks were used to calibrate the mass spectrum. Isotope ratios used for species determinations were as follows: hydrogen, ^{1}H 99.98% and ^{2}H 0.02%; oxygen, ^{16}O 99.76%, ^{17}O 0.04%, and ^{18}O 0.2%; nitrogen, 100% ^{14}N; carbon, ^{12}C 98.9%, ^{13}C 1.1%; sulfur, ^{32}S 95.02%, ^{33}S 0.75%, ^{34}S 4.21%, and ^{36}S 0.02%; copper, ^{63}Cu 69.17% and ^{65}Cu 30.83%.

Total copper in culture medium—Duplicate 1-ml aliquots of culture filtrate were acidified with 5 mol L^{-1} HCl to a final concentration of 1 mmol L^{-1} and stored in acid-washed polycarbonate tubes prior to analysis with GFAAS. Standard additions were used to account for matrix affects. Duplicate samples were also analyzed using inductively coupled plasma-mass spectrometry (ICP-MS) with stable isotope dilution and MgO_2 precipitation with good agreement (Wu and Boyle 1998; Fones pers. comm.).

Competing ligand exchange adsorptive cathodic stripping voltammetry (CLE-CSV)—Cu complexation in culture media was characterized by CLE-CSV using benzoylacetone (bzac) as the competing ligand (Moffett and Brand 1996). In this work, the culture filtrates were divided into 20-ml aliquots in 125-ml Teflon bottles. These were spiked with different concentrations of copper (1–200 nmol L^{-1}) and bzac (1–5 × 10^{-4} mol L^{-1}) and allowed to equilibrate for several hours. The ligands produced by *E. huxleyi* and the bzac compete for the added copper, though bzac is a Cu (II) chelator and would not be expected to compete with thiols for Cu (I). However, this approach should reveal whether Cu is strongly complexed, regardless of redox state, if formation of the Cu (II)-benzoylacetone complex is suppressed relative to ultraviolet-irradiated controls. Moreover, Leal and van den Berg (1998) argued that quantitative information can be obtained about Cu (I) complexes using a Cu (II) competing ligand if certain assumptions are made about rapid Cu (I)/Cu (II) redox equilibria in solution. For the analysis, 10 ml of solution was transferred to a quartz sample cup on the electrode in static mercury drop mode. Instrument settings were as follows: deposition potential, −0.08 V (vs. Ag/AgCl electrode); deposition time, t_d = 1 min; scan range, −0.08 V to −0.5 V; scan rate 10 mV s^{-1}; drop time 0.2 s; pulse height 25 mV.

Results

Intracellular thiols—HPLC-ESI-ITMS analysis of the derivatized extracts of cell homogenates from both the copper-

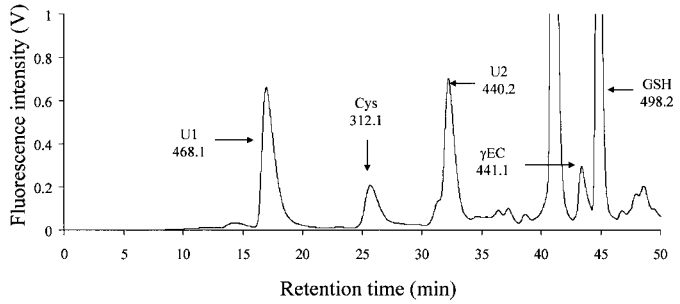

Fig. 1. An HPLC chromatogram (fluorescence vs. time) of a mBBr-derivatized cell extract of a Cu-stressed *E. huxleyi* culture. The identity of various peaks along with the *m/z* of each is indicated on the figure. The dipeptides Arg-Cys and Gln-Cys identified in this article are labeled U1 and U2, respectively. The peak at 40 min is unlabeled mBBr.

treated and non-copper-treated cultures verified the presence of known thiols GSH, Cys, γ-Glu-Cys, and phytochelatin ($n = 2$) in *E. huxleyi* (Fig. 1). Remarkably, two peaks that did not correspond with known standards were present on both the fluorescence trace (Fig. 1) and the ITMS ion current chromatogram (not shown). The mBBr-labeled compounds responsible for these peaks have *m/z* of 468.2 and 440.1, which corresponds to masses of 278.1 and 250.0 for the original unlabeled thiol compounds, assuming each has only one mBBr molecule associated with it. These unidentified compounds, present in both control and copper-treated cultures, were in sufficiently high concentrations in the algal cell extracts to sequentially trap and fragment each with ITMS. A series of MS/MS experiments were performed in which the molecular ion was isolated and fragmented, followed by the isolation and fragmentation of the next largest ion, generating fragmentation profiles up to MS^4. Based on the masses and fragmentation patterns, the compounds were tentatively identified as arginine-cysteine (Arg-Cys) and glutamine-cysteine (Gln-Cys), which we subsequently had synthesized. The mass spectral fragmentation patterns of the synthetic Arg-Cys and Gln-Cys matched those of the unknown peaks, confirming the hypothesized identities (MS^3 profiles shown in Fig. 2). These dipeptides have not been observed in other organisms and have no previously known biological function.

Several geographically distinct clones of *E. huxleyi* were all found to produce intracellular Arg-Cys and Gln-Cys, but the intracellular concentrations during late-exponential growth were highly variable among the strains (Table 1). Gln-Cys varied by close to 10-fold, and a factor of 26 was

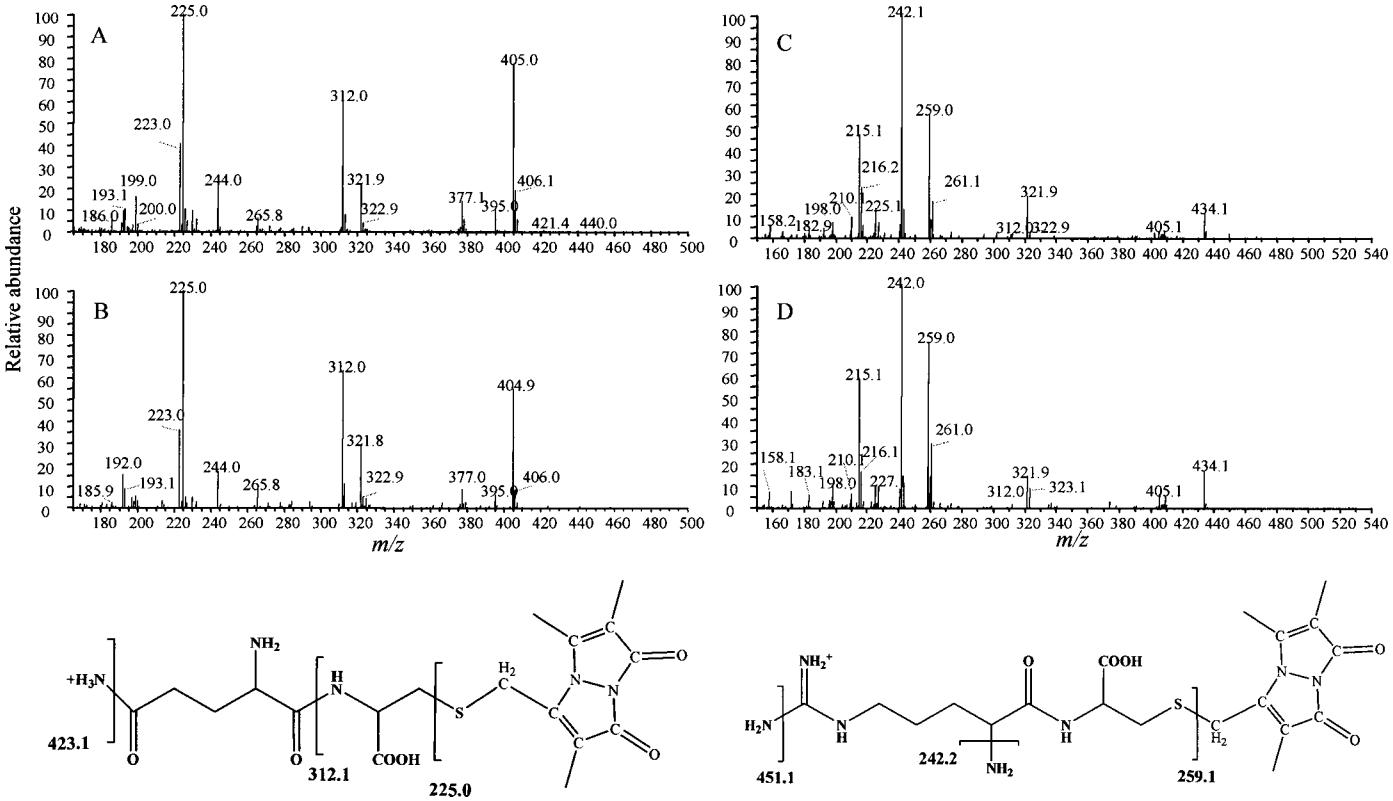

Fig. 2. MS^3 spectrum of mBBr-derivatized thiol-containing compounds: (A) Unknown 2, (B) synthetic Gln-Cys, (C) Unknown 1, and (D) synthetic Arg-Cys. The Gln-Cys fragmentation pathway starts with the Gln-Cys-mBBr M+1 molecular ion (*m/z* = 440.2) and is followed by the trapping and fragmentation of an ion of *m/z* 423.1 (neutral loss of an amine) to yield the MS^3 spectrum, which features the major ion fragments (*m/z* = 405.0, 312.0, and 225.0) shown in panels A and B. The Arg-Cys fragmentation pathway starts with the Arg-Cys-mBBr M+1 molecular ion (*m/z* = 468.2) and proceeds through an ion at *m/z* 451.1 (neutral loss of an amine) to yield a MS^3 mass spectrum that features the major ion fragments (*m/z* = 434.1, 321.9, 261.0, 259.0, 242.1, and 215.1) shown in panels C and D. Structures of derivatized thiols are shown with theorized fragmentation sites. The dipeptide is shown in bold.

Table 1. Particulate thiol concentrations (in μmol [g^{-1} Chl *a*]) in several geographically distinct strains of *E. huxleyi* grown in AQUIL at pCu 13.8 (Price et al. 1988/1989). With the exception of CCMP 373, which was used for the majority of the experiments in this study, strains were provided by B. Palenik and D. Landry. Cells were harvested during late-exponential growth (~700,000 cells ml^{-1}). Values represent single measurements from individual cultures; duplication of some yielded very low variability (±5%).

Strain	Location	Arg-Cys	Gin-Cys	Cys (μmol [g^{-1} Chl *a*])	γEC	GSH
CCMP 373	N. Atlantic	260	370	90	190	700
374-10	N. Atlantic	25	50	65	125	555
CCMP 1516	N. Atlantic	10	160	110	200	510
382-1	Cal. Current	10	35	30	65	285
361-1	Cal. Current	25	75	70	130	610
EH GO	Norway	35	80	155	125	520

observed between the highest and lowest concentrations of Arg-Cys. The strain used in the majority of experiments presented in this study (CCMP 373) contained by far the highest concentrations of both of these thiols. While glutathione concentrations were relatively consistent, varying at most by a factor of 2, intracellular concentrations of γ-Glu-Cys and Cys varied three- to fivefold (Table 1). The variation between strains could not be explained by geographic distribution. A limited selection of marine algae, including diatoms, green algae, and coccolithophores, was also surveyed for Arg-Cys and Gln-Cys production, but only *Geophyscrysa oceanica*, a coccolithophore closely related to *E. huxleyi*, was found to produce small amounts of Gln-Cys, but not Arg-Cys. It appears that these compounds are not widespread among marine phytoplankton, but further work is warranted.

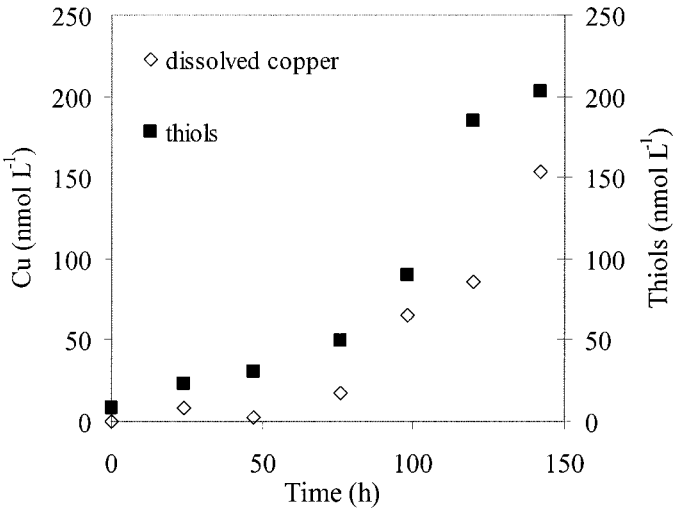

Fig. 3. Total dissolved thiol concentrations and total dissolved copper concentrations in the growth media of an *E. huxleyi* culture over 6 d with daily addition of 50 nmol L^{-1} copper. Thiols were measured using HPLC. Dissolved copper was measured using ICP-MS (*see* Materials and methods). At the final time point, [Arg-Cys] = 75 nmol L^{-1}, [Cys] = 60 nmol L^{-1}, and [Gln-Cys] = 50 nmol L^{-1}. Both dissolved Cu and total thiol measurements represent the average of duplicate samples taken from a single experimental culture.

Dissolved thiols—To stimulate the production of copper ligands by *E. huxleyi*, copper was added to cultures grown in AQUIL with no added chelators, a similar experimental design to that described in Moffett and Brand (1996). As observed in that study, the total dissolved copper concentrations were always lower than the amount added, presumably because of accumulation in the cells, though after 3 d of copper additions, the copper in the growth media increased roughly in proportion to the amount of copper that was added (Fig. 3). Throughout the experiment, both control and copper-treated cultures remained in exponential growth. As copper was added to the culture medium over the course of the experiment, total dissolved thiols were always in excess dissolved copper (Fig. 3). The non–copper-treated control culture showed only minor total thiol exudation over the same time (20–30 nmol L^{-1} over 144 hr). When this experiment was repeated to obtain material for the MALDI-TOF analysis, similar ratios of total thiols to total dissolved Cu were observed (data not shown). HPLC retention times of compounds in the growth media matched those of the particulate thiols identified with the ITMS, and peak identities were verified using standard additions because dissolved concentrations were below the detection limit of the ITMS. Throughout the experiment the newly identified thiols Arg-Cys and Gln-Cys, along with Cys, composed most (~90%) of the dissolved thiol pool (35%, 25%, and 30%, respectively), while GSH, γ-Glu-Cys, and phytochelatins were present at low concentrations or were below detection. The absence of appreciable amounts of phytochelatins, γ-Glu-Cys, and especially glutathione confirms that cell breakage or leakage during filtration cannot be used to explain the apparent exudation, as they make up a sizable fraction of the intracellular thiol pool (Table 1).

Copper speciation and complexation—To identify specific thiol-Cu complexes in the growth media, copper-treated *E. huxleyi* culture filtrate was fractionated using size exclusion chromatography. The majority of the thiols (primarily Cys and Arg-Cys) and copper coeluted in one peak that was collected in several fractions between 10 and 11 min (Fig. 4). These fractions were pooled, lyophilized, and then analyzed using MALDI-TOF MS. Peaks corresponding to the exact masses of protonated Cys-Cu (I) (m/z = 184), $(Cys)_2$-Cu (I) (m/z = 152.5, doubly protonated), and Arg-Cys-Cu (I)-Cys

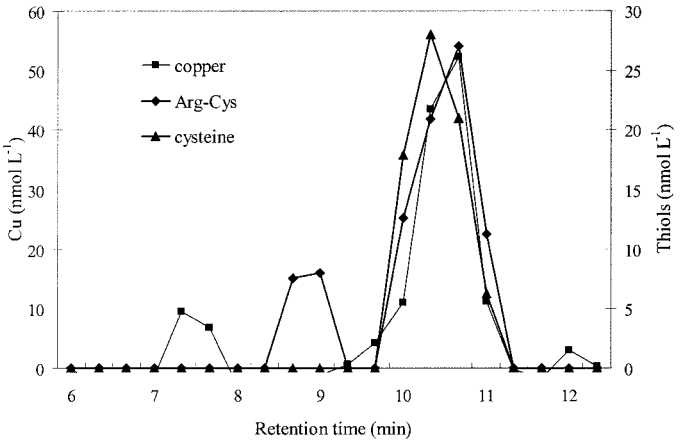

Fig. 4. Size exclusion chromatogram of thiols and copper from the growth media of a copper-exposed *E. huxleyi* culture. The thiols and copper in the collected fractions were measured using HPLC and GFAAS, respectively. [Cu] in culture filtrate = 170 nmol L^{-1}, [Arg-Cys] = 100 nmol L^{-1}, [Cys] = 60 nmol L^{-1}, [Gln-Cys] = 15 nmol L^{-1}. Gln-Cys concentrations were below detection following size-exclusion chromatography.

(m/z = 432) were observed and verified using theoretical isotope ratio profiles (the MS profile of Arg-Cys-Cu (I)-Cys is shown in Fig. 5). Peaks corresponding to free Cys and Arg-Cys were also observed, but both free Gln-Cys and Cu complexes containing Gln-Cys were not observed. The culture filtrate used in this experiment contained little Gln-Cys compared to Arg-Cys and Cys (as compared to that described in Fig. 3); therefore, Gln-Cys complexes may have been below detection. It is also possible that Gln-Cys, which is readily ionized by the softer electrospray technique, does not survive the more energetic MALDI procedure. Minor peaks corresponding to oxidized thiols were also observed. The MALDI-TOF peaks attributed to thiol-Cu (I) complexes and uncomplexed thiols were not present in a similarly treated control culture medium and were lower in intensity when the samples were not lyophilized prior to MS analysis. GSH, γ-Glu-Cys, phytochelatins, and copper complexes of these compounds were not observed in any of the samples.

CLE-ACSV measurements on growth media revealed no detectable Cu chelators, except at the final and highest Cu concentration (153 nmol L^{-1}). At lower Cu concentrations, chelators may have been present, but they were unable to compete for the Cu in solution with the 10^{-4} mol L^{-1} benzoylacetone added. At the final time point, however, the dissolved copper was complexed by a chelator or class of chelators with binding strength (log $K' = 11.6$) comparable to those observed in open ocean waters and in a previous study with *E. huxleyi* (Leal et al. 1999). The concentrations of these ligands (145–160 nmol L^{-1}) were similar to the concentration of copper in the growth media (153 nmol L^{-1}). Since the measured amounts of dissolved thiols are not high enough to support complete Cu complexation via *bis* complexes, we suggest that there may be a mixture of both *mono* and *bis* thiol-Cu complexes (as we observed using MALDI-TOF mass spectrometry), and that there may be other non-thiol-containing ligands present in the culture medium. Another possibility is that our HPLC measurements underestimate the true total thiol concentrations, perhaps because a fraction of the thiols remain bound to Cu and are thus inaccessible to the mBBr probe, despite the addition of excess DTPA prior to derivatization. This is unlikely, though, as acidification of some samples to both pH 3 and pH 1 (*see* Materials and methods) prior to the addition of borate buffer and DTPA yielded nearly identical thiol concentrations to those presented here (data not shown).

A possible explanation for why we did not see strong chelators throughout our experiment is that the competing ligand used, bzac, interacts with the Cu (I)-thiol complexes, displacing and oxidizing the Cu (I) to Cu (II). The bzac is now able to equilibrate with the thiols and Cu (II), and being a stronger Cu (II) ligand, outcompetes the thiols giving the appearance of a lack of strong copper ligands. While possible, this is unlikely. In order for this reaction to occur, bzac would have to bind Cu (I), and being an oxygen donor ligand, has only a weak affinity for a soft B-type metal like

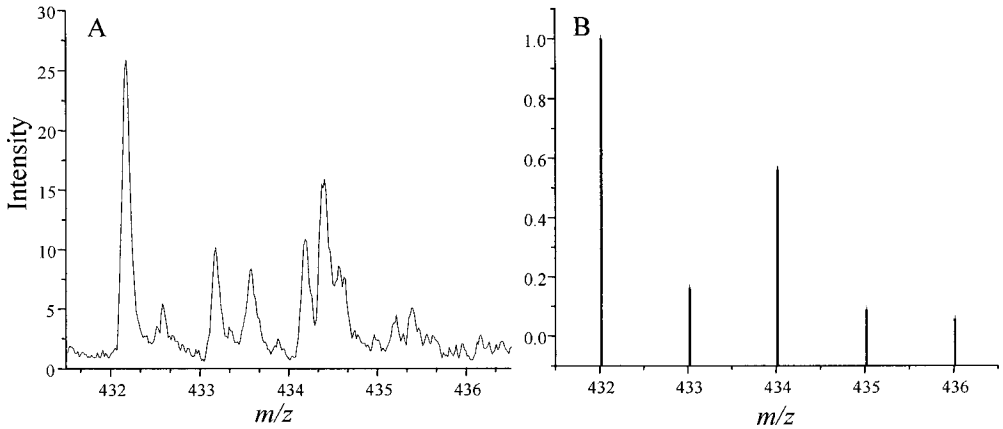

Fig. 5. (A) Representative MALDI-TOF MS profile of the size-fractionated copper-exposed *E. huxleyi* culture filtrate shown with (B) the theoretical Arg-Cys-Cu(I)-Cys isotope profile. For the experimental data (panel A), the intensity of the peaks is relative to the most abundant cation in the sample. For the theoretical isotope ratio profile (panel B), the most abundant isotope is given an intensity of 1, with other isotopes plotted in proportion.

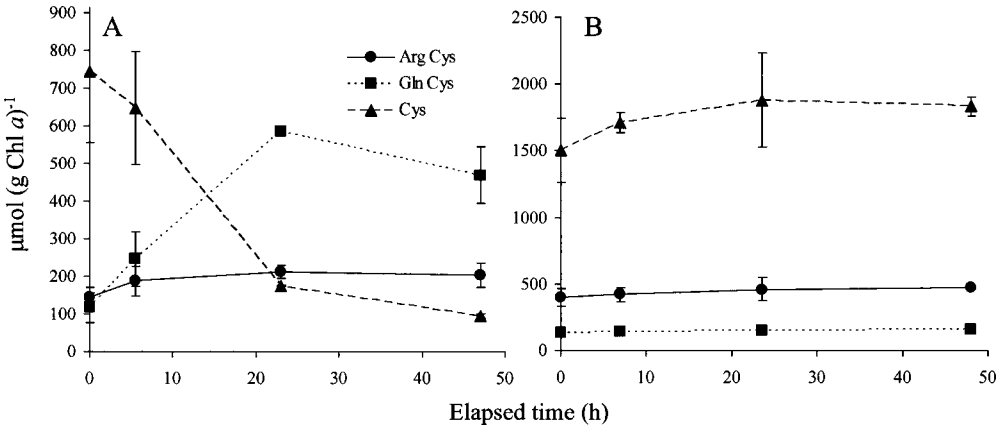

Fig. 6. Particulate concentrations of Cys, Arg-Cys, and Gln-Cys in (A) nitrogen- and (B) phosphate-limited *E. huxleyi* following the addition of 100 μmol L^{-1} $NaNO_3$ or 10 μmol L^{-1} NaH_2PO4, respectively. Cultures were grown in nitrogen- or phosphate-deficient AQUIL until stationary phase prior to nutrient addition. Glutathione concentrations were similar and remained constant during both experiments. Error bars represent the range of duplicate measurements of the particulate thiols in single cultures.

Cu (I). Additionally, this mechanism would not explain why we observed strong ligands at the conclusion of the experiment unless there was an efflux of non-thiol Cu (II) ligands by *E. huxleyi* just prior to that analysis.

N- and P-limited cultures—To determine if the nitrogen-rich thiols Arg-Cys and Gln-Cys are involved in nitrogen storage or assimilation, cultures were grown in N-deficient media and treated with 100 μmol L^{-1} sodium nitrate shortly after the end of exponential growth. Over 24 hr, intracellular Gln-Cys and Arg-Cys concentrations increased by 400% and 50%, respectively, while cysteine was rapidly depleted in proportion to the molar concentrations of Arg-Cys and Gln-Cys formed (Fig. 6A). Conversely, little variation in the concentrations of these thiols was observed in phosphate-limited cultures following phosphate addition, though the culture conditions resulted in remarkably high cysteine and Arg-Cys concentrations (Fig. 6B). In both experiments, glutathione concentrations were similar ($\sim$700 μmol (g^{-1} Chl *a*)) and remained constant following the addition of the limiting nutrient (data not shown).

Discussion

The novel thiols identified here could account for a portion of the strong copper-binding ligands observed in surface ocean waters, especially in *E. huxleyi*–dominated regions. The strong ligands responsible for the nearly complete complexation of Cu in surface seawater have been assumed to be Cu (II) chelators (Ross et al. 2003; Vachet and Callaway 2003), although appreciable levels of Cu (I) are present in seawater (Moffett and Zika 1988). Organic complexation of copper by thiols has been shown to stabilize Cu (I) in seawater, and some experiments have shown that thiols may be indistinguishable from Cu (II) ligands (Leal and van den Berg 1998), which analysts using a variety of speciation techniques have generally assumed. Here we have demonstrated that Cu (I)-thiol complexes are formed through biological activity, though the mechanism of formation is uncertain.

We hypothesize that the Cu (I) complexes are formed intracellularly and then exuded into the medium, as $(thiol)_n$-Cu (I) complexes are thermodynamically favored in high-thiol, low-Cu environments such as blood plasma (Tran-Ho et al. 1997) or cell cytoplasm. Such a mechanism is consistent with the stoichiometric export of Cd and $n = 2$ phytochelatin by *Thalassiosira weisflogii* (Lee et al. 1996). Additionally, Croot et al. (2003) have shown that *Synechococcus* sp. (WH7803) actively efflux Cu following uptake, and they have suggested that the Cu is exported as a ligand-Cu complex. Conversely, free ligands could be released into the growth medium to decrease Cu bioavailability, effectively blocking uptake. Given the multifaceted chemistry of thiols and Cu in seawater, several routes of extracellular complex formation are possible; therefore, further investigation into the mechanism of formation is needed.

Strong ligands were observed in similar concentrations to dissolved copper at the completion of the experiment using CLE-ACSV. Appreciable amounts of strong ligands were not measured prior to this, but chelators weaker than the added competing ligand (benzoylacetone, log $K' = 10.7$ for the *bis* complex) may have been present. While $(thiol)_2$-Cu (I) complexes are likely very stable in seawater (Leal and van den Berg 1998), the weaker (and possibly undetectable using our CLE-ACSV technique) thiol-Cu(I) complexes may predominate in our experiments. We do know from the MALDI-TOF data that there was a mixture of *mono* and *bis* complexes at the end of the experiment. It will require further investigation to determine the relative amounts and strengths of *mono* and *bis* thiol-Cu complexes and whether other non–thiol-containing chelators are contributing to the pool of strong ligands measured via CLE-ACSV.

In a previous study of copper-induced exudation by *E. huxleyi*, Leal et al. (1999) reported the production of a compound or class of compounds that are electrochemically ac-

tive, producing a well defined peak at ~−0.65 V (vs. Ag/AgCl) in cathodic stripping voltammetry. They have argued that these peaks are probably generated by thiols, since they are very similar to peaks produced in the presence of glutathione. Our findings support their conclusion that thiols are exuded in response to Cu, but it is clear from our study that glutathione is not the principal exudates, as has been hypothesized.

An efficient sequestration and export mechanism involving thiol-Cu (I) complexes could explain the high copper tolerance of *E. huxleyi* (Brand et al. 1986), especially in combination with its high DMSP content, which might provide a scavenging mechanism for ·OH radicals generated by redox reactive copper (Sunda et al. 2002). Given the widespread success of *E. huxleyi* and its capacity to form large blooms, the exudation of copper-binding ligands by this algal species could also have dramatic effects upon copper speciation in large areas of the open ocean.

In addition to the proposed role in copper detoxification, Arg-Cys and Gln-Cys, rich in nitrogen and structurally similar to the nitrogen storage compound cyanophycin found in cyanobacteria (Wingard et al. 2002), may be used by *E. huxleyi* for nitrogen storage. The response of intracellular Gln-Cys upon the addition of nitrogen indicates a role in nitrogen assimilation. The rapid synthesis of Gln-Cys would require a rapid supply of glutamine, a product of glutamine synthetase (GS), the enzyme responsible for inorganic nitrogen assimilation. There are two kinetically distinct isoforms of GS in *E. huxleyi* (Maurin and Le Gal 1997*b*), and nitrogen starvation can induce a shift to the isoform exhibiting a half saturation constant (K_m) for NH_4 higher than that of other algae in nitrogen-limiting environments (Maurin and Le Gal 1997*a*). Our data indicate that in *E. huxleyi,* Gln-Cys may serve as a compound to temporarily store newly synthesized glutamine, allowing *E. huxleyi* to take advantage of transitory periods of enhanced nitrogen availability. While only minor increases in Arg-Cys were observed during this experiment, prolonged exposure to high nitrogen results in elevated intracellular concentrations (Fig. 6B and other unpublished results), implying that this compound may also play a storage role.

Despite a reportedly low uptake rate for nitrate (Riegman et al. 2000) and the lack of a vacuole that permits nutrient storage, *E. huxleyi* can be very successful in semioligotrophic regions where nitrogen is low (Iglesias-Rodriguez et al. 2002). The intracellular concentrations of Arg-Cys and Gln-Cys (0.5–5 mmol L^{-1}) are similar to those of glycine betaine and are 10 times higher than those of homarine, two nitrogeneous osmolytes in *E. huxleyi* (Keller et al. 1999) and may provide an accessible pool of nitrogen-rich amino acids. Such a dynamic and readily available pool of nitrogen may be an adaptation that allows *E. huxleyi* to compete in semioligotrophic environments.

References

AHNER, B. A., AND F. M. M. MOREL. 1999. Phytochelatins in microalgae, p. 1–31. *In* Progress in phycological research, V. 13. Biopress.

———, L. P. WEI, J. R. OLESON, AND N. OGURA. 2002. Glutathione and other low molecular weight thiols in marine phytoplankton under metal stress. Mar. Ecol. Prog. Ser. **232:** 93–103.

ANDREAE, M. O., AND P. J. CRUTZEN. 1997. Atmospheric aerosols: Biogeochemical sources and role in atmospheric chemistry. Science **276:** 1052–1058.

BRAND, L. E., W. G. SUNDA, AND R. R. L. GUILLARD. 1986. Reduction of marine phytoplankton reproduction rates by copper and cadmium. J. Exp. Mar. Biol. Ecol. **96:** 225–250.

COALE, K. H., AND K. W. BRULAND. 1988. Copper complexation in the Northeast Pacific. Limnol. Oceanogr. **33:** 1084–1101.

CROOT, P. L., B. KARLSON, J. T. VAN ELTEREN, AND J. J. KROON. 2003. Uptake and efflux of ^{64}Cu by the marine cyanobacterium *Synechococcus* (WH7803). Limnol. Oceanogr. **48:** 179–188.

———, J. W. MOFFETT, AND L. E. BRAND. 2000. Production of extracellular Cu complexing ligands by eucaryotic phytoplankton in response to Cu stress. Limnol. Oceanogr. **45:** 619–627.

FERNANDEZ, E., P. BOYD, P. M. HOLLIGAN, AND D. S. HARBOUR. 1993. Production of organic and inorganic carbon within a large-scale coccolithophore bloom in the northeast Atlantic Ocean. Mar. Ecol. Prog. Ser. **97:** 271–285.

IGLESIAS-RODRIGUEZ, M. D., C. W. BROWN, S. C. DONEY, ET AL. 2002. Representing key phytoplankton functional groups in ocean carbon cycle models: Coccolithophorids. Global Biogeochem. Cycles **16:** art num 1100.

KELLER, M. D., R. P. KIENE, P. A. MATRAI, AND W. K. BELLOWS. 1999. Production of glycine betaine and dimethylsulfoniopropionate in marine phytoplankton. I. Batch cultures. Mar. Biol. **135:** 237–248.

LEAL, M. F. C., AND C. M. G. VAN DEN BERG. 1998. Evidence for strong copper (I) complexation by organic ligands in seawater. Aquat. Geochem. **4:** 49–75.

———, M.T.S.D. VASCONCELOS, AND C. M. G. VAN DEN BERG. 1999. Copper-induced release of complexing ligands similar to thiols by *Emiliania huxleyi* in seawater cultures. Limnol. Oceanogr. **44:** 1750–1762.

LEE, J. G., B. A. AHNER, AND F. M. M. MOREL. 1996. Export of cadmium and phytochelatin by the marine diatom *Thalassiosira weissflogii.* Environ. Sci. Technol. **30:** 1814–1821.

MANN, E. L., N. AHLGREN, J. W. MOFFETT, AND S. W. CHISHOLM. 2002. Copper toxicity and cyanobacteria ecology in the Sargasso Sea. Limnol. Oceanogr. **47:** 976–988.

MAURIN, C., AND Y. LE GAL. 1997*a.* Glutamine synthetase in the marine coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae): Regulation of activity in relation to light and nitrogen availability. Plant Sci. **122:** 61–69.

———, AND ———. 1997*b.* Isoforms of glutamine synthetase in the marine coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae). Compar. Biochem. Physiol. B Biochem. Mol. Biol. **118B:** 903–912.

MOFFETT, J. W. 1995. Temporal and spatial variability of Cu complexation by strong chelators in the Sargasso Sea. Deep-Sea Res. **42:** 1273–1295.

———, AND L. E. BRAND. 1996. Production of strong extracellular Cu chelators by marine cyanobacteria in response to Cu stress. Limnol. Oceanogr. **41:** 388–395.

———, ———, P. L. CROOT, AND K. A. BARBEAU. 1997. Cu speciation and cyanobacterial distribution in harbors subject to anthropogenic Cu inputs. Limnol. Oceanogr. **42:** 789–799.

———, AND R. G. ZIKA. 1988. Measurement of copper (I) in surface waters of the subtropical Atlantic and Gulf of Mexico. Geochim. Cosmochim. Acta **52:** 1849–1857.

NAPP, J. M., AND G. L. HUNT. 2001. Anomolous conditions in the south-eastern Bering Sea 1997: Linkages among climate, weather, ocean, and biology. Fish. Oceanogr. **10:** 61–68.

PRICE, N. M., G. I. HARRISON, J. G. HERING, R. J. HUDSON, P. M. V. NIREL, B. PALENIK, AND F. M. M. MOREL. 1988/1989. Prep-

aration and chemistry of the artificial algal culture medium Aquil. Biol. Oceanogr. **6:** 443–461.

RIEGMAN, R., W. STOLTE, A. A. M. NOORDELOOS, AND D. SLEZAK. 2000. Nutrient uptake and alkaline phosphatase (EC 3:1:3:1) activity of *Emiliania huxleyi* (Prymnesiophyceae) during growth under N and P limitation in continuous cultures. J. Phycol. **36:** 87–96.

ROSS, A. R. S., M. G. IKONOMOU, AND K. J. ORIANS. 2003. Characterization of copper-complexing ligands in seawater using immobilized copper (II)-ion affinity chromatography and electrospray ionization mass spectrometry. Mar. Chem. **83:** 47–58.

SHOAF, W. T., AND B. W. LIUM. 1976. Improved extraction of Chlorophyll *a* and *b* from algae using dimethyl sulfoxide. Limnol. Oceanogr. **21:** 926–928.

SOURNIA, A. [ED.]. 1978. Phytoplankton manual. United Nations Educational, Scientific and Cultural Organization.

SUNDA, W., D. J. KIEBER, R. P. KIENE, AND S. A. HUNTSMAN. 2002. An antioxidant function for DMSP and DMS in marine algae. Nature **418:** 317–320.

TRAN-HO, L., P. MAY, AND G. T. HEFTER. 1997. Complexation of copper (I) by thioamino acids. Implications for copper speciation in blood plasma. J. Inorg. Biochem. **68:** 225–231.

TYRRELL, T., P. M. HOLLIGAN, AND C. D. MOBLEY. 1999. Optical impacts of oceanic coccolithophore blooms. J. Geophys. Res. **104:** 3223–3241.

VACHET, R. W., AND M. B. CALLAWAY. 2003. Characterization of Cu (II)-binding ligands from the Chesapeake Bay using high-performance size-exclusion chromatography and mass spectrometry. Mar. Chem. **82:** 31–45.

VAN DEN BERG, C. M. G. 1984. Determination of the complexing capacity and conditional stability constants of copper (II) with natural organic ligands in seawater by cathodic stripping voltammetry of copper-catechol complex ions. Mar. Chem. **15:** 1–18.

WINGARD, L. L., S. R. MILLER, J. M. L. SELLKER, E. STENN, M. M. ALLEN, AND A. M. WOOD. 2002. Cyanophycin production in a phycoerythrin-containing marine *Synechococcus* strain of unusual phylogenetic affinity. Appl. Environ. Microbiol. **68:** 1772–1777.

WU, J., AND E. A. BOYLE. 1998. Determination of iron in seawater by high-resolution isotope dilution inductively coupled plasma mass spectrometry after $Mg(OH)_2$ coprecipitation. Anal. Chim. Acta **367:** 183–191.

Received: 19 November 2003
Accepted: 11 May 2004
Amended: 31 May 2004