

Copper-induced release of complexing ligands similar to thiols by *Emiliana huxleyi* in seawater cultures

M. Fernanda C. Leal

Oceanography Laboratories, University of Liverpool, Liverpool L69 3BX, U.K.; and LAQUIPAI, Chemistry Department, Faculty of Science, University of Porto, 4150 Porto, Portugal

M. Teresa S. D. Vasconcelos

LAQUIPAI, Chemistry Department, Faculty of Science, University of Porto, 4150 Porto, Portugal

*Constant M. G. van den Berg*¹

Oceanography Laboratories, University of Liverpool, Liverpool L69 3BX, U.K.

Abstract

Marine microalgae (*Emiliana huxleyi*) were grown in seawater enriched only with nitrogen and phosphorus, without control of free metal concentrations using synthetic chelators. Complexing ligands and thiol compounds were determined by cathodic stripping volt-ammetry. Copper was added to these cultures, and ligands were produced in response to the copper addition. Parallel measurements of thiols showed that glutathione and other unidentified thiols (electrochemically similar to thioacetamide) were produced by the algae at rates and concentrations similar to those of the complexing ligands. Smaller amounts of thiols were produced when ligands including thiols were added to the culture. The results indicate that thiols can account for a major part or most of the copper-complexing ligands produced by these algae. Furthermore, a feedback mechanism exists in which the production of thiol-type complexing ligands is controlled by the free copper concentration, production already being stimulated by an increase of $[\text{Cu}^{2+}]$ from 0.4 to 1.5 pM. Incubations with added exudates, thiols, and salicylaldehyde (SA) showed much reduced copper toxicity even though copper uptake was increased by the exudates and the SA.

Culture experiments have shown that marine and freshwater phytoplankton release complexing ligands into culture medium (e.g., van den Berg et al. 1979; Imber and Robinson 1983), which may be important because the availability of metals to algae is known to depend on their chemical composition (e.g., Brand et al. 1983). Therefore, the interaction of phytoplankton with trace metals is reciprocal: the biota affect the trace metal chemistry and the trace metals affect the biota.

Using marine algal cultures where the metal chemistry was controlled by a synthetic chelator (usually ethylenediaminetetraacetic acid [EDTA]), the biological uptake of copper has been shown to be related to the cupric ion concentration, $[\text{Cu}^{2+}]$ (e.g., Brand et al. 1986); toxicity occurs at pM levels of Cu^{2+} . The concentration of Cu^{2+} is typically lowered by 2–3 orders of magnitude by organic complexation in seawater to a $[\text{Cu}^{2+}]$ of 0.03–0.3 pM, which is thought to be sufficiently high to meet algal nutritional requirements but is below copper toxicity levels (Sunda 1995).

At such low free ionic concentrations, it is likely that the bioavailable fraction includes all species in thermodynamic equilibrium with Cu^{2+} , i.e., the inorganic species CuCO_3^0 , $\text{Cu}(\text{CO}_3)^{2-}$, CuOH^+ , and $\text{Cu}(\text{OH})_2^0$ and perhaps part of the organic complexes, which can dissociate in the diffusion layer surrounding the organisms.

Organic complexation can reduce metal availability for kinetic reasons if the metal uptake is restricted to the inorganic metal species. Although metal availability to algae in cultures is controlled by EDTA when this molecule is in great excess (suggesting that the inorganic metal is the bioavailable fraction), certain organic copper complexes (e.g., lipid-soluble complexes) are themselves taken up (Florence et al. 1992). This kind of metal availability is masked in the presence of excess EDTA in conventional cultures, which tends to outcompete all other metal-complexing ligands. For this reason it is interesting to culture algae without the control of metal speciation by EDTA, instead relying on possible production of chelating agents by the organisms to provide a medium suitable for growth. One drawback is that the metal speciation cannot then be predicted from the known medium composition but must be determined experimentally.

Little is known about the nature of the ligands. The stronger complexing ligands cause a problem because no suitable candidates are known for such stable complexation of Cu^{2+} in seawater. Copper(I) (Cu^+) is very strongly complexed by thiol compounds, and the redox reactions of copper are sufficiently fast to allow reduction of copper(II) to copper(I) within minutes when a common thiol like glutathione is add-

¹ Corresponding author.

Acknowledgments

This research was partly supported by the MERLIM project of the European Union (MAS3-CT95-0005) and by a grant from the NERC (PRIME, GST/02/1058) for which this is publication 90. The Portuguese Foundation for Science and Technology provided a Ph.D. scholarship to M.F.C.L. (PRAXIS XXI Program—BD/5044/95), which included a stay at Liverpool University. Assistance with CHN analyses by Doris Angus and general laboratory assistance by Carmel Murphy are gratefully acknowledged.

ed to seawater (Leal and van den Berg 1998). Recent work (Leal and van den Berg 1998) has shown that the usual speciation techniques do not differentiate between copper complexation as copper(I) or copper(II), and copper(I)-complexing ligands are detected as if they bind copper(II) with conditional stability constants ($K'_{Cu(II)L}$) on the order of 10^{11} – 10^{12} . Therefore, the copper speciation in cultures and natural waters may be dominated by the copper(I)-binding complexing ligands. Copper(I) complexation and thiol compounds should therefore be included when copper complexation is investigated.

The source of the organic ligands in seawater is not well known. These ligands may be produced by organisms by excretion or cell lysis or by breakdown of existing organic material. Copper-complexing ligands are known to be produced by marine fungi (Sunda and Gessner 1989) and cyanobacteria (*Synechococcus*), with complex stabilities ($\log K'_{Cu(II)L}$ 11–12) similar to that of the strong ligand in the Sargasso Sea (Moffett et al. 1990). Organic complexation of copper in the oceanic water column is generally highest near the productivity maximum and decreases with depth below the vernal mixed layer (Bruland et al. 1991). Organic ligand concentrations vary from 100 nM (van den Berg 1984) or higher (Manping et al. 1990) in coastal waters to 10–20 nM in oceanic waters (Buckley and van den Berg 1986; Coale and Bruland 1988).

In summary, there is evidence that organic ligands that complex with copper (and other trace metals) are produced by marine microorganisms, but little is known about the composition of these ligands. The fraction of the total dissolved metal that is bioavailable is controversial and poorly defined.

In this work, we investigated the production of exudates (organic ligands and thiol compounds) in cultures of the marine microalga *Emiliania huxleyi* (Coccolithophores) in un-supplemented seawater, enriched only with nitrate and phosphate (without trace metals, chelators, or vitamin enrichments), to simulate the natural marine environment. The aim of the study was to establish whether there is a relationship between thiol exudation, copper speciation, and copper availability (toxicity). The copper concentration in the seawater was varied, and the copper uptake and speciation were monitored. The production of exudates (ligand concentrations and conditional stability constants) was followed by titrations with copper and detection by cathodic stripping voltammetry (CSV) with ligand competition. Specific thiols (glutathione) and thiols with a response similar to thioacetamide and thiourea were quantified by CSV. Ligand and thiol additions were used to investigate possible feedback mechanisms.

Materials and methods

Reagents—Water was purified by reverse osmosis (Milli-RO 10 Plus) followed by ion-exchange (Milli-Q, Millipore). This water was used for reagent preparation and rinsing. Reagents were AnalaR grade (BDH) unless indicated otherwise. Both HCl and NH_3 (Merck) were purified by isothermal distillation. Copper standard solutions were prepared by

dilution of an atomic absorption spectrometry standard solution (Spectrosol grade) with 0.01 M HCl.

An aqueous stock solution containing 0.01 M salicylal-doxime (SA) was prepared in 0.1 M HCl and stored at 4°C. A pH buffer containing 1 M boric acid (Aristar grade) and 0.35 M NH_3 was irradiated with ultraviolet (UV) light for 3 h to remove organic matter; 100 μ l of this buffer in 10 ml seawater gave a pH of 8.35 (NBS pH scale).

Working standards of glutathione (Biochemicals), thioacetamide, thiourea, and folic acid (Sigma) were prepared daily from 0.1 M stock solutions that were prepared weekly in water and stored at 4°C. A standard of chlorophyll *a* (Chl *a*) (Fluka, Biochemika) was prepared by dissolving 1 mg of standard Chl *a* in 5 ml of 90% acetone. A lugol solution was prepared dissolving 1 g of iodine (M&B, Pronalys) and 2 g of potassium iodide in 20 ml of water. LB medium was prepared dissolving 10 g of Bactrotryptone, 5 g of yeast extract, and 10 g of NaCl per liter of water. The pH was adjusted to 7 with NaOH. The medium was preserved in sealable containers and autoclaved.

The seawater used for these experiments originated from the North Atlantic Ocean (*Prime* Cruise; 46°50'N, 19°95'W; collected at 3 m depth). The water was filtered (0.1 μ m Nuclepore) and stored in a 50-liter high-density polyethylene (HDPE) container.

Decontamination of the equipment and cultures—The culture media for the algae were sterilized by filtration (with a 0.1- μ m polycarbonate membrane, Millipore) using a peristaltic pump (Minipuls 3, Gilson). The filters, Erlenmeyer flasks, polycarbonate bottles, and plastics (pipette tips, filtration system, etc.) were previously acid cleaned (1 M HCl during 1 week) and rinsed with water. All materials were sterilized by microwave (AVM 705, Philips) at 600 W for 5 min (2, 1, and 2 min at intervals of 30 s); autoclaving was not used to avoid trace metal contamination via the steam and to prevent possible precipitation of carbonate species (Price et al. 1989). The presence of bacteria in the cultures was tested using LB medium (Standard bacteriological non-selective growth medium). Aliquots of 0.1 ml were transferred to LB medium (10 ml), and during 2 weeks (the normal growth period) no clouds of bacteria were observed, suggesting that the cultures were not significantly contaminated with bacteria. Bottles of polycarbonate (Nalgene) were cleaned by soaking with hot water and detergent. They were then rinsed with water and filled with 1 M HCl for 1 week, rinsed again, and filled with 1 M HCl for another week. Finally, the bottles were filled with 0.01 M HCl and stored in resealable bags. HDPE sample bottles (Nalgene) were cleaned by soaking with hot water and detergent, soaked for 1 week in 50% HCl (GPR grade) and then in 2 M HNO_3 , and then filled with 0.01 M HCl and stored in sealed bags. All sample manipulations were carried out in a laminar flow hood with HEPA filtered air.

Stock cultures of *E. huxleyi*—Stock unialgal and axenic cultures of *E. huxleyi* (Lohmann PCC 92 and 92d, isolated by Hay and Muller, Plymouth Marine Laboratory) were grown in synthetic seawater (ca. 5×10^3 cells ml^{-1}). The synthetic seawater was prepared by dissolution of 23.38 g

NaCl, 0.75 g KCl, 4.93 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.07 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2.19 g $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.17 g NaHCO_3 , and 0.025 g H_3BO_3 in 1 liter of water. Major nutrients, e.g., nitrate (N) and phosphate (P), trace metals, and vitamins were added to this synthetic seawater to *f*/2 levels (Stein 1973). In an Erlenmeyer flask, 1 ml of stock algae was inoculated into 250 ml of the medium using pipette tips with an air filter (Aeroseal Gold filter, Fisher Brand). The initial concentration of algae in the medium was approximately 5×10^3 cells ml^{-1} . The cultures were incubated in a Illuminated Cooled Incubator (Mercia Scientific) with continuous (24 h) illumination at 15°C, until the stationary phase was reached.

Cultures of E. huxleyi in enriched natural seawater—These cultures were grown in natural seawater enriched only with N and P added to final concentrations of 176 μM and 7.26 μM , respectively (equivalent to *f*/10 concentrations). The enriched seawater was sterilized by filtration into a 1-liter polycarbonate bottle and inoculated with 1 ml of the *f*/2 stock culture on day 7 of growth (exponential phase), providing a 1:1,000 dilution of the concentrations of trace metals, EDTA, and vitamins and of the number of cells in the *f*/2 medium. In these cultures, the initial concentration of algae was approximately 0.5×10^3 cells ml^{-1} .

Determination of chlorophyll and cell numbers in the algal cultures—The concentration of Chl *a* was determined spectrophotometrically using a UV/Vis Spectrometer (Unicam UV 2) or fluorometrically using a Luminescence Spectrometer (Perkin Elmer LS-5) every 2–3 d according to the method of Parsons et al. (1984). Ten to 100 ml of algal culture, depending on the day of growth and type of culture, was filtered using a water pump and glass microfiber filters (GF/C 70 mm, Whatman).

Cell numbers were counted every 2–3 days, using an Inverted Biological Microscope (Wild M40, Wild Heerbrugg) according to established methods (Lund et al. 1958). Volumes of 0.05–1 ml (stock cultures) or 0.2–5 ml (natural seawater cultures), depending on the culture age, were placed in sedimentation tubes and diluted to 2 ml (if the volume was less), and 20 μl of Lugol's solution was added. The cells were left to settle for 1 h (2 h if 5 ml of culture was used) and then counted.

Determination of N and P in the cultures—N and P were determined using a UV/Vis spectrometer (Unicam UV 2) in filtered culture aliquots, which were stored frozen at –20°C until analysis. For the N determination, the samples were passed through a cadmium column (column containing cadmium filings coated with metallic copper) to reduce the nitrate to nitrite. The algal weight was determined by freeze drying (Modulyo, Edwards) the filters.

Speciation of copper in the cultures—The concentrations of labile copper ($[\text{Cu}]_{\text{labile}}$) and dissolved copper ($[\text{Cu}]_d$) were determined in filtered subsamples by CSV both before and after inoculation of the seawater. The cultures were gravity filtered to avoid cell damage using a polycarbonate (Sartorius) filter apparatus (0.4 μm polycarbonate membrane, Millipore, acid cleaned). Voltammetric equipment consisted of

an Autolab voltammeter (Ecochemie) connected to a Metrohm 663-VA electrode stand (HMDE; drop size approximately 0.52 mm^2). The reference electrode was double-junction Ag|saturated AgCl in 3 M KCl|3 M KCl, and the counter electrode was glassy carbon. During the adsorption step, solutions were stirred by a polytetrafluoroethylene (PTFE) rod rotating at 2,500 rpm. The voltammeter was controlled by an IBM-AT compatible computer.

The $[\text{Cu}]_{\text{labile}}$ was determined by CSV in the presence of 0.01 M borate buffer and 25 μM SA, after a purging time of 5 min with O_2 -free nitrogen, using a 60-s adsorption step (t_d) and a deposition potential (E_d) of –0.2 V while stirring. The potential scan was carried out in a negative direction after a 10-s quiescence period from –0.15 V to –0.90 V using the square-wave modulation. The scan rate was 20 mV s^{-1} , modulation amplitude was 25 mV, and the square-wave frequency was 50 Hz. Each scan was repeated three times, and the procedure was repeated after addition of copper standard to calibrate the sensitivity.

The $[\text{Cu}]_d$ was determined using a higher concentration of SA (50 μM) (giving a detection window centered at $\alpha = 10^{6.3}$) and with $E_d = -1.1$ V, a large overpotential to include organically complexed copper in the analysis (thiol complexes dissociate under this condition; only complexes with $K'_{\text{CuL}} > 10^{14}$ and that are electrochemically inert could still retain copper). Comparative analyses were carried after UV digestion of acidified aliquots (pH 2.2, by addition of 10 μl of 6 M HCl per 10 ml). Samples were UV irradiated for 3 h with a 600-W high-pressure mercury-vapour lamp and were positioned concentrically in Teflon-capped silica tubes (acid cleaned) of ca. 30 ml volume; 6 M NH_3 was used to approximately neutralize the pH prior to the reagent addition.

Copper in the algae ($[\text{Cu}]_{\text{algae}}$) was determined after filtration (0.4- μm pore size filter) of the culture medium (150 ml). The filters were ashed at room temperature using an oxygen plasma asher in a PT 7150 RF Plasma Barrel Etcher (Bio Rad, Microscience Division) evacuated to a pressure of 0.03 mbar. The residue in the silica vessels was recovered by rinsing with 1 ml of 0.1 M HCl and several portions of 1 ml of water (to a final volume of 10 ml). Copper was then determined by CSV. Blank filters were taken through the same procedure, and the Cu contamination was insignificant (<1%).

The copper complexing ligand concentrations (C_L) and K'_{CuL} were determined by copper titrations with detection of labile copper by CSV. Borate pH buffer (0.01 M) and SA ligand (5 μM) were then added to 120 ml of filtered culture medium. Aliquots of 10 ml were pipetted into 11 polystyrene vials (28-ml Sterilins) also containing added copper in the range of 0–120 nM. The solutions were equilibrated overnight (12–15 h) prior to the labile copper determinations. The sensitivity was obtained from the linear portion of the titration where all the ligands were saturated with copper. The sensitivity was corroborated by comparison with the slope obtained by further copper additions at the high end of the titration, after the $[\text{Cu}]_{\text{labile}}$ had been measured. The volt-ammetric cell was not rinsed between aliquots to maintain cell conditioning to the copper and SA concentrations. The aliquots were measured sequentially from 0 to 120 nM

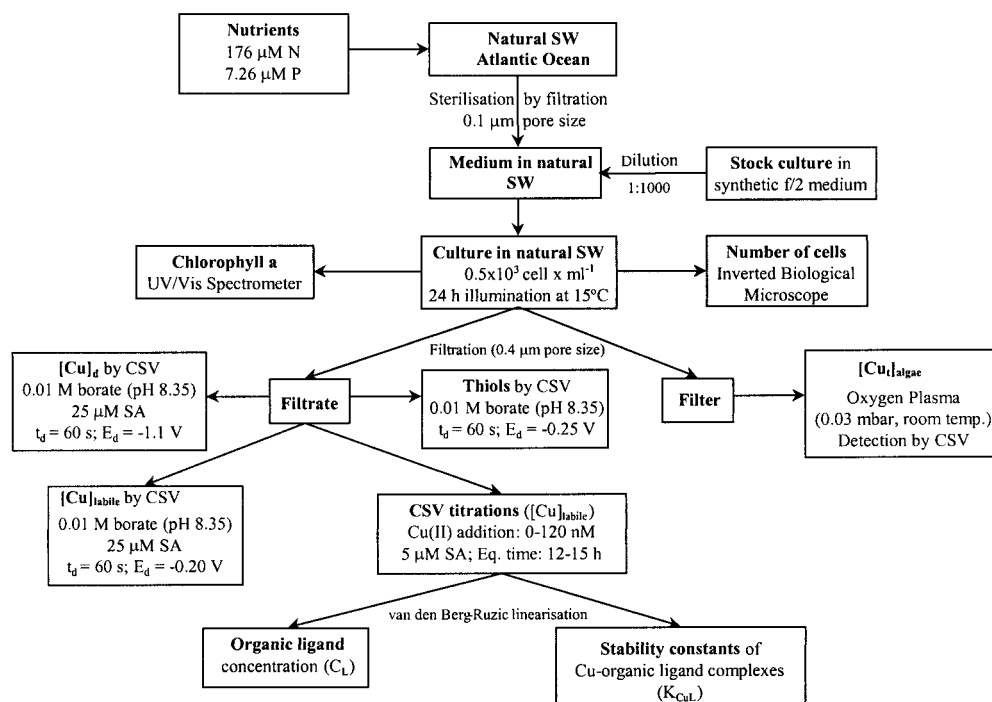


Fig. 1. Experimental design used in this work.

added copper. The Sterilin tubes were rinsed with water between titrations, and the same order of tubes was maintained.

Determination of thiols—Thiols were determined by CSV in filtered culture medium using the method of Al-Farawati and van den Berg (1997); a standard voltammetric cell was used rather than the flow cell recommended for sulfide because the thiols are not affected by the decrease of the peak height characteristic for sulfide. The adsorption time was 60 s at $E_d = 0.25$ V. The potential scan was from -0.15 to -0.90 V, using the square-wave modulation (50 Hz). Each scan was repeated three times, and the procedure was repeated after addition of thiol standard (glutathione or thioacetamide) for calibration. The glutathione peak was located at -0.58 V, and the thioacetamide peak (indicative of other thiol compounds) was at -0.68 V.

A summary of the methods is presented in Fig. 1.

Calculations—The $[Cu]_{labile}$ in the titrations is defined by the copper, which equilibrates with and is bound by the added SA. $[Cu]_{labile}$ includes the inorganic copper (because it equilibrates with the copper added during calibration) and any copper released from organic complexes in competition with the added SA:

$$[Cu]_{labile} = \sum [CuSA_x] + [Cu]_{inorg} = (\alpha_{CuSA} + \alpha_{Cu(II)})[Cu^{2+}]. \quad (1)$$

Here the contribution of inorganic copper(I) is ignored because its concentration is much less than that of inorganic copper(II) and cannot be calculated accurately because the redox potential of the system is not known.

Ligand concentrations were calculated using the Ruzic/van den Berg linearization of the data (Ruzic 1982; van den

Berg 1982) as implemented previously for copper and SA (Campos and van den Berg 1994):

$$\begin{aligned} & [Cu]_{labile}/[Cu(II)L] \\ &= [Cu]_{labile}/C_L + (\alpha_{Cu(II)} + \alpha_{CuSA})/(C_L K_{Cu(II)L}), \quad (2) \end{aligned}$$

where the conditional stability constant $K'_{Cu(II)L} = [Cu(II)L]/[Cu^{2+}][L']$, $\alpha_{Cu(II)}$ is the α coefficient for inorganic complexation of copper(II), C_L is the concentration of the unknown ligand L, $[L']$ is the concentration of L not complexed by copper, and α_{CuSA} is the α coefficient for complexation of copper(II) by SA:

$$\alpha_{CuSA} = K'_{CuSA}[SA'] + \beta'_{Cu(SA)_2}[SA']^2 \quad (3)$$

(Campos and van den Berg 1994). C_L was calculated from the slope of the linear least-squares regression, whereas $K_{Cu(II)L}$ was obtained from the y-axis intercept.

Preliminary experiments using thiols indicated that glutathione binds copper as copper(I) in seawater (Leal and van den Berg 1998); this finding has not been confirmed for other thiol compounds, but an analogous reaction is likely. However, it is not certain that all the ligands exuded by the algae are thiols. For this reason, it is presently not known whether the copper is complexed as copper(I) or copper(II) in these cultures and in seawater generally. It may then be preferable not to make any prior assumptions about the oxidation state of copper in its complexes with L and to use a conditional stability constant of the type

$$K'_{CuL} = [CuL]/([Cu'][L']), \quad (4)$$

where $[Cu']$ is the concentration of copper not complexed by L. In this constant the oxidation state of copper is left

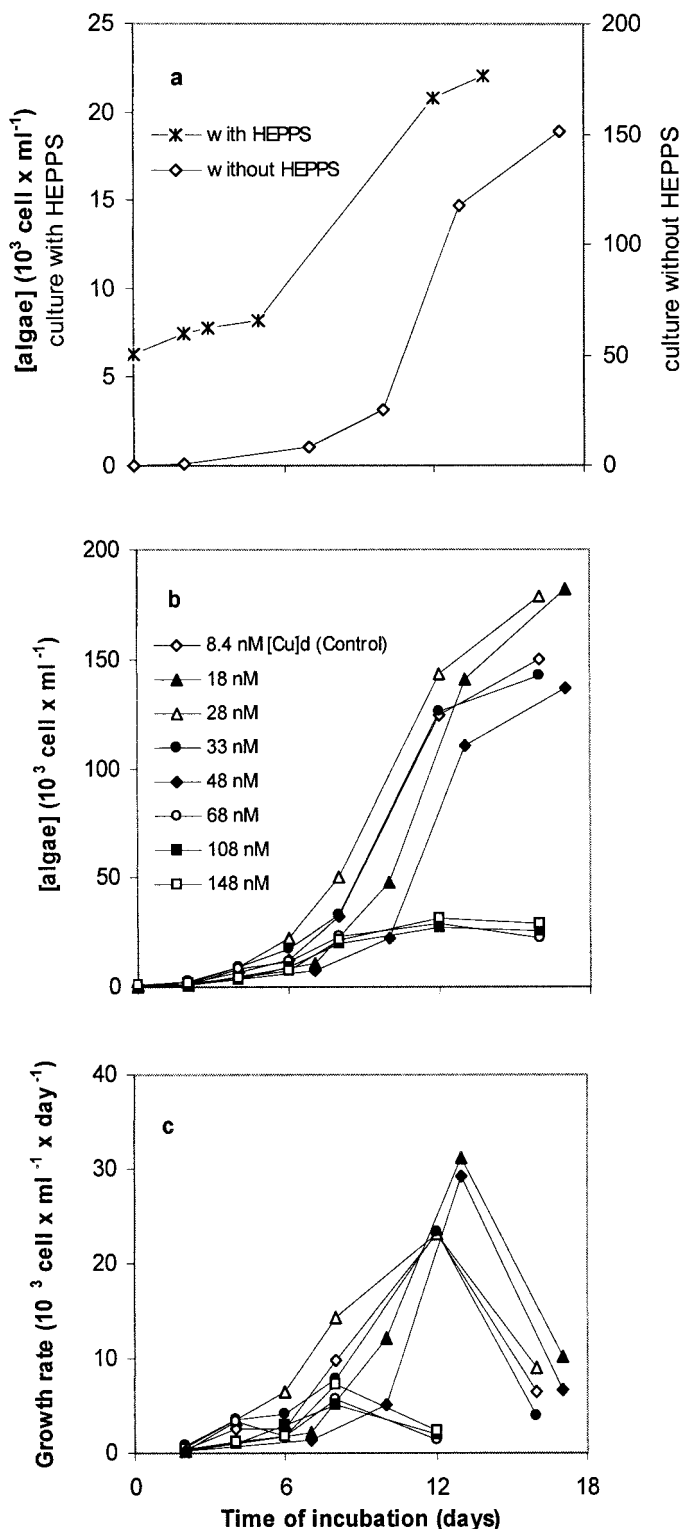


Fig. 2. Growth of *E. huxleyi* in enriched natural seawater. Comparison of growth for the control culture with and without HEPPS buffer (a), and effect of the copper concentration ($[Cu]_d$) on the growth (b) and growth rates (c) of the cultures.

Table 1. Nitrate (N) and phosphate (P) in the course of the *E. huxleyi* cultures.

Culture age (d)	Cell number (10 ³ cell ml ⁻¹)	Algae (pmol cell ⁻¹)		Filtrate		
		N	P	N (μM)	P (μM)	N/P
0*				176	7.26	24
1	1.40			174	7.61	23
7	69.6	0.59	0.068	134	2.99	8.7
11	162	0.45	0.046	101	0.18	9.8

open. However, because of the nature of the CSV method the complex stability is determined with respect to the competing Cu(II)-binding ligand SA, and the constant determined is $K'_{Cu(II)L}$ as if copper occurs as copper(II). A value for K'_{CuL} is calculated from $K'_{Cu(II)L}$ using the following relationship:

$$K'_{CuL} = K'_{Cu(II)L} / \alpha_{Cu(II)} \quad (5)$$

For comparative purposes and speculatively, this constant can be converted to one valid for copper(I) complexation ($K'_{Cu(I)L} = [Cu(I)L] / ([Cu^+][L])$) using

$$K'_{Cu(I)L} = K'_{CuL} \times \alpha_{Cu(I)}, \quad (6)$$

where $\alpha_{Cu(I)}$ is the α coefficient for inorganic complexation of Cu^+ . This equation assumes that the redox couples reach equilibrium quickly (Leal and van den Berg 1998) and that to a first approximation the redox potential of the system is such that there are equal concentrations of inorganic Cu(I) and Cu(II) (at 100 nM H_2O_2 $[Cu(I)]_{inorganic} \sim 0.1 [Cu(II)]_{inorganic}$ so the estimated $K'_{Cu(I)L}$ could easily be underestimated by a factor of ≥ 10). A value of 36, valid for pH 8.3 seawater (our experimental condition), was calculated for $\alpha_{Cu(II)}$, and a value of 1.4×10^5 was calculated for $\alpha_{Cu(I)}$.

Because the major part of inorganic copper is Cu(II), the contribution of copper as copper(I) to inorganic copper was neglected in the mass balance, and the concentration of Cu^{2+} was calculated using

$$[Cu^{2+}] = \{-b + (2b - 4ac)^{1/2}\} / 2a \quad (7)$$

where $a = \alpha_{Cu(II)} K'_{Cu(II)L} C_L$, $b = \alpha_{Cu(II)} + K'_{Cu(II)L} C_L - K'_{Cu(II)L} [Cu]_d$, and $c = -[Cu]_d$. The inorganic copper concentration was then calculated using

$$[Cu]_{inorg} = [Cu^{2+}] \alpha_{Cu(II)}, \quad (8)$$

and the organic copper concentration was calculated using

$$[Cu]_{org} = ([Cu(II)L]) = [Cu]_d - [Cu]_{inorg}. \quad (9)$$

Results

Comparison of different methods used to evaluate the growth of E. huxleyi—Growth of *E. huxleyi* in f/2 medium was evaluated from the concentrations of Chl *a* and from the cell numbers. The two parameters gave very close correlation (correlation coefficient ≥ 0.99) during the exponential phase, suggesting that either method was acceptable. Repeated experiments showed that the exponential growth phase started normally after 3–4 days of incubation and was

Table 2. Copper speciation in the algal cultures as a function of culture age. Speciation was calculated using the average value for the stability constant for each culture.

Time (d)	[Algae] (10 ³ cell ml ⁻¹)	[Cu] _f (nM)	[Cu] _{algae} (fmol cell ⁻¹)	[Cu] _d (nM)	[Cu] _{labile} (nM)	C _L (nM)	Log K' _{Cu(II)L}	[Cu ²⁺] (pM)	[Cu] _{inorg} (pM)	[Cu] _{org} (nM)	CuL (%)
Control				8.4							
0	0.28	0.04	0.14	8.36	6.55	34.2	12.10	0.43	15.3	8.34	99.8
2	0.65	0.38	0.58	7.98	4.63	43.7	11.86	0.29	10.6	7.97	99.9
7	8.07	1.74	0.22	6.62	4.19	58.6	12.13	0.17	6.04	6.61	99.9
10	25.4	2.09	0.08	6.27	3.37	68.7	11.72	0.13	4.76	6.27	99.9
Mean ± SD							11.95 ± 0.20				
Control				18.4							
0	0.28	0.04	0.14	18.4		34.2	12.10	1.52	54.8	18.3	99.7
2	0.70	6.93	9.90	11.4	6.45	47.0	11.57	0.42	15.2	11.4	99.9
7	11.1	8.08	0.73	10.3	5.93	61.8	12.09	0.26	9.46	10.3	99.9
10	47.4	8.74	0.18	9.62	4.45	87.1	11.59	0.16	5.89	9.61	99.9
Mean ± SD							11.84 ± 0.30				
Control				33.4							
0	0.28	0.04	0.14	33.4		34.2	12.10	25.2	907	32.5	97.2
2	0.57	13.9	24.4	19.5	14.8	54.4	11.57	0.73	26.4	19.5	99.9
7	7.09	16.4	2.31	17.0	10.7	83.2	11.75	0.34	12.2	17.0	99.9
10	22.2	20.1	0.91	13.3	6.24	89.1	11.95	0.23	8.29	13.3	99.9
Mean ± SD							11.84 ± 0.23				

maintained for 3–4 days, after which the stationary phase occurred. The results were similar to those obtained previously for the same algae and medium (Plavsic et al. 1997).

Growth of E. huxleyi in enriched natural seawater—Preliminary experiments were carried out to evaluate whether *E. huxleyi* would grow satisfactorily in seawater supplemented only with N and P. The initial N and P concentrations were varied between those of f/2 and f/10 (f/2 concentrations diluted by a factor of 5), and the effect of omitting the addition of metals and vitamins was tested without EDTA. At f/10 concentrations (176 μM N and 7.26 μM P), greatest growth was attained without the metal and vitamin addition; apparently, there was no requirement for the vitamins, and the metal supplement probably was toxic in the absence of EDTA.

Comparative cultures with and without 0.01 M HEPES buffer (pH 8) showed (Fig. 2a) that growth was higher (almost 10-fold) in the absence of the buffer. Apparently, buffer with this pH can have a deleterious effect, in agreement with previous work suggesting that a buffer of similar pH (HEPES) can affect algae by increasing copper toxicity, albeit that that work was carried out at very high copper concentrations (Lage et al. 1996). The pH of the *E. huxleyi* cultures without pH buffer did not vary significantly over the course of the experiments, presumably because of the simultaneous production of organic matter and calcite coccoliths, so the subsequent culture media did not include a pH buffer.

The growth of *E. huxleyi* in N/P-enriched seawater was slower than that in f/2 medium. The exponential phase started after 6–7 d of incubation, was maintained for 5–6 d, and then decreased. The growth trend was similar to that in f/2 medium, but the growth was delayed by 3 d. Furthermore, the final yield was much less, approximately 10% of that

achieved in the f/2 medium, and the final cell numbers (up to about 2×10^5 cells ml⁻¹) were similar to those found in a natural bloom of *E. huxleyi* (van der Wal et al. 1995). In view of a low final P concentration, these cultures may have ultimately become P limited. The slower growth may have been due to factors other than copper, in view of the absence of EDTA and vitamins.

This experiment was repeated three times to assess the reproducibility of these cultures. The relative standard deviation was never higher than 8% (at the beginning of the growth) or 5% (at the end of the growth).

Variation of the N/P concentrations in the medium during the growth of E. huxleyi—The N and P concentrations were measured in the culture medium (filtrate of a control culture) on the first day of the incubations (before growth was initiated), on day 7 (at the beginning of the exponential phase), and on day 11 (end of the exponential phase). These concentrations decreased significantly with culture age (Table 1): from 174 to 101 μM N and from 7.61 to 0.18 μM P. The low final P concentration suggests that the yield of the culture was controlled by P limitation. The initial N/P ratio in the medium was 23. In the cells, the N/P ratio was nearly constant during the incubation period, at 8.7 in the beginning and 9.8 at the end of the exponential phase. The amount of N taken up per cell at the end of the exponential phase (0.45 pmol N cell⁻¹) was similar to that taken up per cell (0.44 pmol cell⁻¹, determined by elemental analyzer) by an *E. huxleyi* culture in “normal” f/2 medium, suggesting that the cultures did not become N limited. Nevertheless, the N/P ratio in these *E. huxleyi* cultures was lower than a more normal ratio of 16 for marine phytoplankton (Sunda 1995).

Effect of copper on the growth of E. huxleyi and production of exudates—In parallel experiments, *E. huxleyi* from a

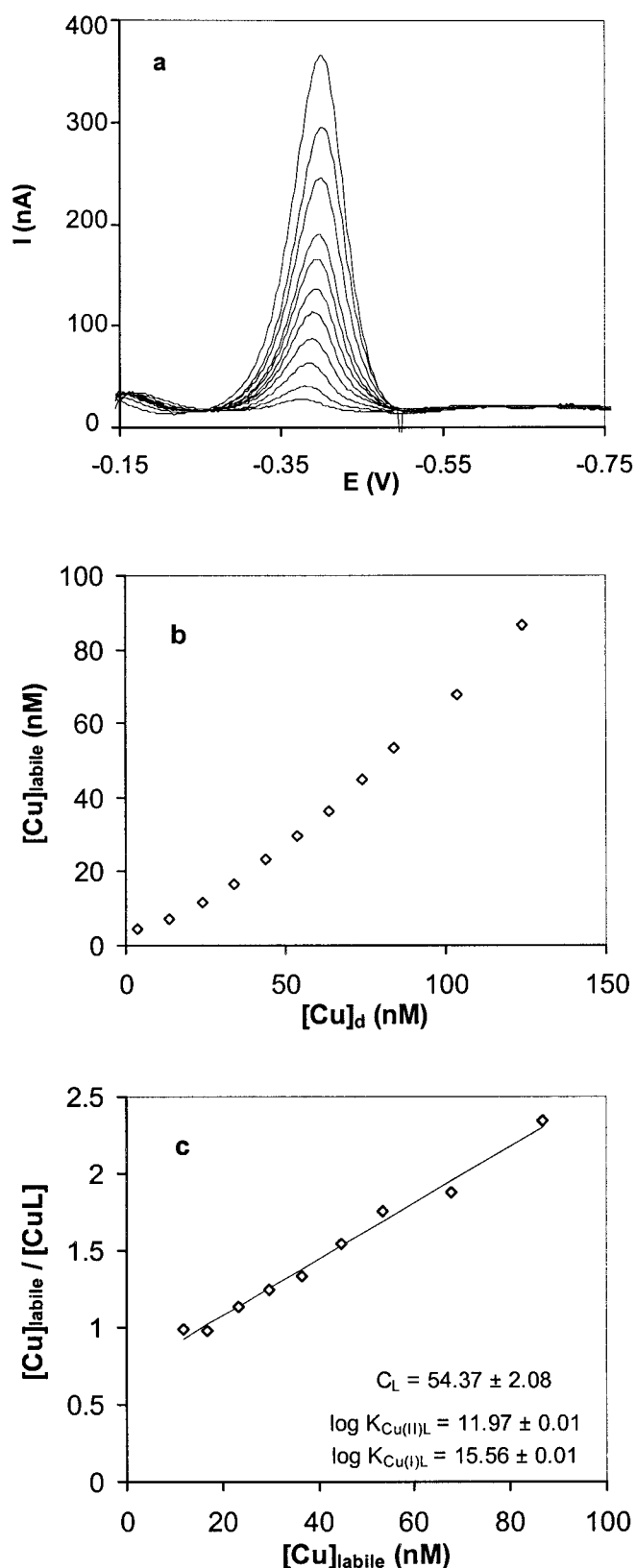


Fig. 3. Example of a copper-complexing ligand titration in an *E. huxleyi* control culture after 7 days of incubation: voltammograms of the titration (a); labile copper as a function of dissolved copper (b); and linearization of the data (c).

single stock culture was inoculated into N/P-enriched seawater (f/10 level) with and without added copper (18, 28, 33, 48, 68, 108, and 148 nM). The metal concentrations before the copper addition were 8.4 nM $[Cu]_d$, 11 nM $[Zn]_d$, and 2 nM $[Fe]_d$, and the ligand concentration was 34 nM C_L . The concentration of other metals that can compete with copper were not determined, but they are known to occur in the open sea at low concentrations: i.e., 4 nM Mn, 0.1 nM Cd, and 0.05 nM Pb (Ahrland 1988). This seawater was used as control medium. Because the composition of the other incubations differed only in the $[Cu]_d$, the differences observed in the biological response of *E. huxleyi* can be considered copper effects.

The inoculation with algae caused significant transfer of iron and EDTA from the f/2 medium, giving final concentrations of 12 nM EDTA and 14 nM $[Fe]_d$. Calculations of the copper speciation at the range of C_L found before and during the algae incubations and at the range of $[Cu]_d$ studied showed that the percentage of copper complexed with EDTA was insignificant ($\leq 0.3\%$). At 14 nM $[Fe]_d$, it is not likely that the cultures were iron limited, which was confirmed in separate experiments (Boye and van den Berg unpubl.).

A 3-h period was allowed after the copper additions to the seawater before inoculation with algae to allow chemical equilibria to be established; full equilibrium is established within 3 h among copper, thiols, and other ligands in seawater (Leal and van den Berg 1998).

Growth was slightly enhanced in the presence of 18 and 28 nM copper as compared with cultures with 8.4 nM copper, whereas at copper concentrations of ≥ 33 nM growth decreased systematically (Fig. 2b,c). The lower growth at 33 nM than at 18 nM copper suggests that this level was no longer optimal for the algae, which were not able to control effectively the cellular copper levels by the release of organic ligands (i.e., the defence mechanism partially failed).

The concentrations of $[Cu]_d$, $[Cu]_{labile}$, $[Cu]_{algae}$, C_L , and the thiol compounds were measured in three of the cultures (control and 18 and 33 nM copper).

Copper uptake by the algae—The dissolved copper concentration in the cultures decreased with culture age, more so at higher copper concentration (Table 2). Control experiments using seawater without algae showed that these losses were not due to adsorption on the culture bottle. In the seawater containing 18 and 33 nM copper, the decrease was 40–44% in the first 2 days of incubation, levelling off after that. The decrease in the labile copper concentration was greater than that of the dissolved copper concentration because of increased complexation by the exudates (Table 2). The copper in the algae (retained by the filter) approximately balanced that lost from the seawater (within an error of $<10\%$). This finding confirmed that the copper adsorption on the polycarbonate culture vessels was negligible.

The copper associated with the algae may have been either taken up or adsorbed on the cell walls, which could act as ligand (Gonzalez-Davila et al. 1995) (adsorption on the cell walls is unlikely in view of the strong complexation necessary to compete against the dissolved ligands). The adsorbed fraction was estimated by washing the cells on the filter (10 min) with 3×10^{-3} M EDTA (Knauer et al. 1997) at various

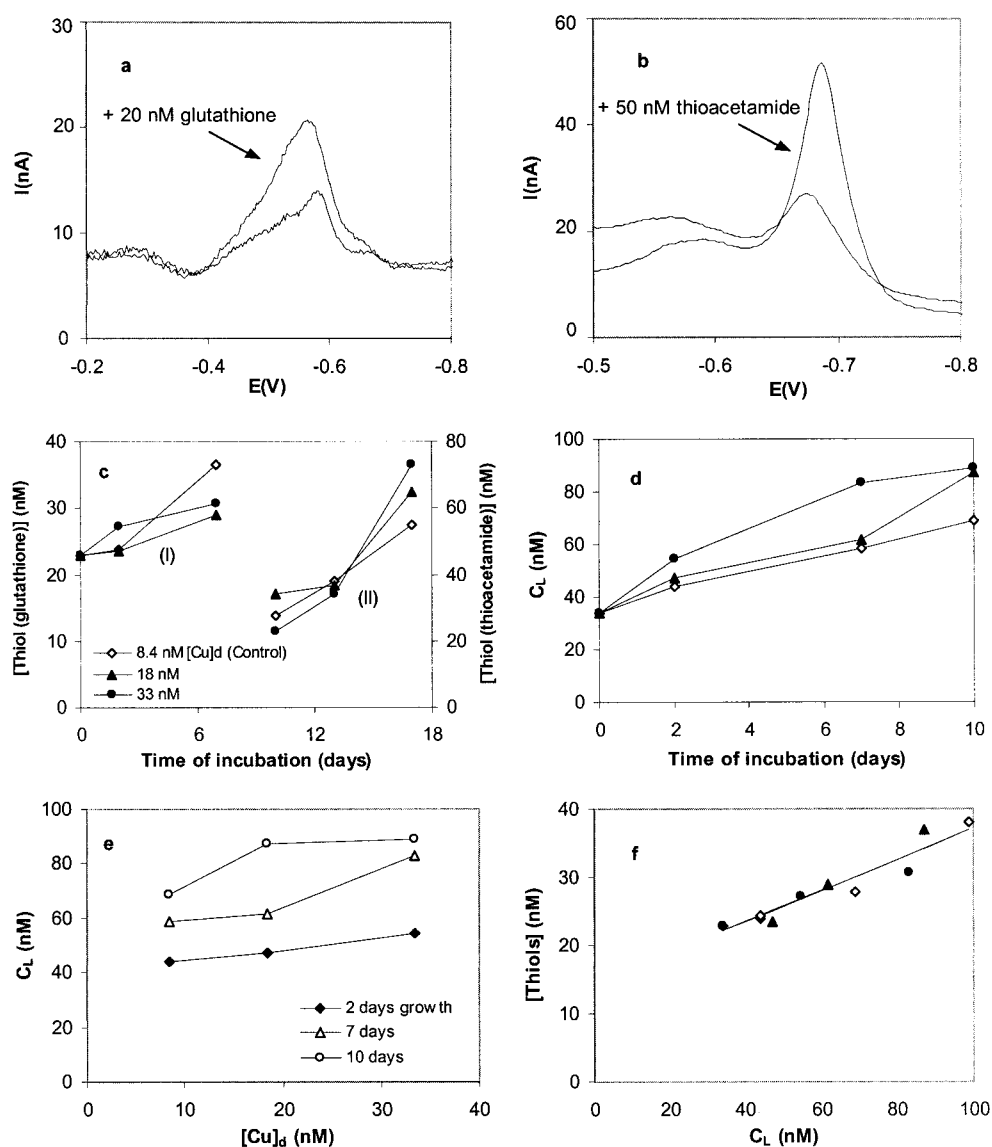


Fig. 4. Thiol compounds produced in *E. huxleyi* cultures. Volt-ammogram of a control culture after 2 d incubation (a) before (1) and after (2) addition of 20 nM glutathione, $E_p = -0.58$ V, and after 7 d incubation (b) before (1) and after (2) addition of 50 nM thioacetamide, $E_p = -0.68$ V. Thiol concentrations (c) were determined through calibration with glutathione (I) and thioacetamide (II). C_L as a function of culture age (d) and as a function of the copper concentration (e) ($[Cu]_d$). The relationship between the concentration of specific thiols and C_L is also shown (f).

copper concentrations; only a small fraction of the algal-bound copper was released, indicating that most (86–96%) was intracellular.

The copper concentration per algal cell ($[Cu]_{\text{algal}}$) was evaluated from $[Cu]_{\text{algal}}$ and from cell densities. The amount of copper per cell decreased exponentially during algal growth, although the overall cellular copper content increased (Table 2) because of the dilution of the fixed amount of copper over an increasing number of cells; the rate of increase in the cell numbers was greater than the rate of uptake of copper, causing the copper-per-cell-concentration to decrease. The data indicate that the algae were able to grow with copper levels varying between 0.5 and 25 fmol

cell⁻¹ ($5\text{--}250 \times 10^{-16}$ mol cell⁻¹). Algal growth improved when the initial copper concentration was raised from 8.4 to 18 nM (where the maximum $[Cu]_{\text{algal}}$ was 9.9 fmol cell⁻¹ compared with a maximum uptake of 0.58 fmol cell⁻¹ at 8.4 nM copper). But algal growth decreased again when the initial copper concentration was raised to 33 nM (maximum $[Cu]_{\text{algal}} = 24$ fmol cell⁻¹). These data suggest that the available copper concentration in the original seawater (8.4 nM) was less than optimal, whereas 33 nM copper was greater than optimal for *E. huxleyi*.

Release of complexing ligands by the algae—During the culture experiments, the C_L (34 nM background level) in-

creased greatly because of production and releases by the algae. The titration of one of the cultures, illustrating the copper scans, the increase in the $[\text{Cu}]_{\text{labile}}$ as a function of the $[\text{Cu}]_d$, and the linearization of the data, is shown in Fig. 3. The data fit a single complexing ligand, showing that the copper complexation (in the tested $[\text{Cu}]_d$ range) is dominated by a single ligand.

The C_L increased gradually with culture age (Table 2, Fig. 4d), reaching 68 nM (control, 8.4 nM $[\text{Cu}]_d$), 87 nM (18 nM $[\text{Cu}]_d$) and 89 nM (33 nM $[\text{Cu}]_d$) after 10 days growth. Because most of the ligands were produced before and during the exponential growth phase, the ligands must have been released from intact cells as a form of exudation.

The C_L increased with the copper concentration in the culture medium (stimulation occurred already at the lowest copper addition: $[\text{Cu}]_d = 18$ nM) at a very low initial $[\text{Cu}^{2+}]$ of 1.5 pM, suggesting that the production was produced in response to or stimulated by the copper addition. Such a response has been observed before for cultures of *Dunaliella tertiolecta* (Gonzalez-Davila et al. 1995) and cyanobacteria (Moffett and Brand 1996), suggesting that the release of ligands may be a general mechanism of resistance to excessive metal levels.

The complex stability did not vary significantly throughout the culture experiments (average $\log K'_{\text{CuL}} = 10.3 \pm 0.3$), and it was similar to that for the ligands originally present in the seawater before the inoculation with algae, indicating that the organic ligands produced by the algae in the culture were functionally similar to those originally present in the seawater.

Complexation of copper by the excreted ligands and possible relationships with algal growth—The chemical speciation of copper in the cultures was calculated from the determined concentrations of Cu, L, and the K'_{CuL} . Because of the ligand production, the fraction of copper occurring as inorganic copper species ($[\text{Cu}]_{\text{inorg}}$) decreased from 15.3 pM at inoculation to 4.8 pM after 10 days ($[\text{Cu}]_d = 8.4$ nM) and from 907 pM to 8.3 pM ($[\text{Cu}]_d = 33$ nM) (Table 2).

Our data demonstrate that *E. huxleyi* growth was optimal at an initial $[\text{Cu}]_{\text{inorg}}$ of 55 pM ($[\text{Cu}]_d = 18$ nM and $[\text{Cu}^{2+}] = 1.5$ pM), whereas 15 pM $[\text{Cu}]_{\text{inorg}}$ ($[\text{Cu}]_d = 8.4$ nM and $[\text{Cu}^{2+}] = 0.4$ pM) was less than optimal, and 907 pM of initial $[\text{Cu}]_{\text{inorg}}$ ($[\text{Cu}]_d = 33$ nM and $[\text{Cu}^{2+}] = 25$ pM) began to be toxic.

The complexing ligands released by the algae in seawater containing a typical $[\text{Cu}]_d$ of 5 nM tend to buffer the $[\text{Cu}]_{\text{inorg}}$ at around 10 pM ($\alpha_{\text{CuL}} = 500$ calculated for 20-nM ligands with a $\log K'_{\text{CuL}} = 10.3$). The ligands would have to be nearly saturated with copper before the $[\text{Cu}]_{\text{inorg}}$ becomes much greater than this. In our experiments, copper inhibition occurred once the $[\text{Cu}]_d$ exceeded the C_L (34 nM in the seawater used here), causing the $[\text{Cu}]_{\text{inorg}}$ to be much greater than 10 pM. For instance, growth was delayed at a dissolved copper concentration of 48 nM (Fig. 2), causing initially $[\text{Cu}]_{\text{inorg}} = 14$ nM and $[\text{Cu}^{2+}] = 0.4$ nM. The smaller copper addition to 33 nM ($[\text{Cu}]_{\text{inorg}} = 907$ pM and $[\text{Cu}^{2+}] = 25$ pM) caused the ligand production to be stimulated, but growth was only marginally less than at 28 nM copper. An unusual resistance of *E. huxleyi* of up to 0.6 nM $[\text{Cu}^{2+}]$ observed

previously (Brand et al. 1986) lies well above the range found in the present study to be suitable for growth.

Identification of the organic ligands produced in the cultures—Some organic compounds can be identified by CSV if they adsorb on the electrode and can be reduced. This group includes several compounds of biological importance, such as folic acid, glutathione, cysteine (Le Gall and van den Berg 1993), and thiol compounds generally (Al-Farawati and van den Berg 1997). Folic acid and glutathione occur in the oceanic water column, indicating their possible importance to the marine system (Le Gall and van den Berg 1998).

During the CSV measurements of copper in the seawater, two additional voltammetric peaks became increasingly apparent with culture age. The first peak, at approximately -0.58 V, was coincident with that for glutathione (Fig. 4a). This peak was apparent in the volt-ammograms of the cultures up to an age of 7 d, after which it became masked by other unresolved peaks. The glutathione peak increased with culture age and was greatest in the control culture (i.e., without copper addition) (Fig. 4c). This result suggests that the algae released exudates such as glutathione with time.

A second identifiable peak, at -0.68 V (Fig. 4b), was detected in cultures of ≥ 10 d and was indicative of thiol compounds (and free sulfide) generally. The peak was not due to free sulfide because if it were its signal would have decreased continuously (Al-Farawati and van den Berg; 1997). The volt-ammetric sensitivity was calibrated using thioacetamide, but several other thiol compounds give the same response. The concentration of these thiol compounds also increased with culture age (such as glutathione) (Fig. 4c) but was greatest in the culture with the highest initial $[\text{Cu}]_d$ tested (33 nM). A plot of the total concentration of thiol compounds (glutathione and others) as a function of the initial $[\text{Cu}]_d$ in the cultures (Fig. 4e) shows the covariation of thiol concentration with $[\text{Cu}]_d$, suggesting that these compounds were produced possibly in response to the copper stress.

The increase in thiol concentrations paralleled that in C_L (Fig. 4f) ($r^2 = 0.96$). The concentration of each individual thiol (such as glutathione or thioacetamide) was $\sim 0.5 C_L$, and the combined concentrations of glutathione and thiols were similar to C_L . The similarity of the detected thiol and ligand concentrations may be fortuitous because the thiol compound used for the calibrations (thioacetamide) may not be a good analogue for the thiols occurring in the cultures. It is therefore not surprising that the C_L was not identical to the thiol concentrations. The similar magnitude and covariation of the ligand and thiol concentrations strongly suggest that these thiol compounds, specifically glutathione and thiols with a CSV behavior similar to that of thioacetamide, may be major contributors to the organic copper-binding ligands released by *E. huxleyi*.

Other compounds thought to be released by algae in cultures include phytochelatin (Ahner and Morel 1995; Scarano and Morelli 1996) and lumichrome (a photolysis product of riboflavin) (Morelli and Scarano 1994). Lumichrome has a peak potential of -0.68 V, similar to that of thioacetamide. We did not test this compound, but its peak shape probably

Table 3. Effect of ligand additions on the growth of *E. huxleyi* and the production of complexing ligands. Copper speciation is shown as a function of culture age. The ligands detected in the culture containing SA are in addition to the SA itself.

Time (d)	[Algae] (10 ³ cell ml ⁻¹)	[Cu] _{r,algae} (nM)	[Cu] _{algae} (fmol cell ⁻¹)	[Cu] _d (nM)	[Cu] _{labile} (nM)	C _L (nM)	Log K' _{Cu(II)L}	[Cu ²⁺] (pM)	[Cu] _{inorg} (pM)	[Cu] _{org} (nM)	CuL (%)
Control											
0	0.56	0.08	0.14	8.26	4.55	33.8	11.71	0.43	15.3	8.24	99.8
2	1.43	1.00	0.70	7.26	2.29	41.3	12.18	0.28	10.1	7.25	99.9
4	3.14	1.25	0.40	7.01	1.43	46.4	12.09	0.23	8.44	7.00	99.9
6	12.6	1.70	0.14	6.56	1.00	54.2	12.01	0.18	6.52	6.55	99.8
8	29.8	2.25	0.076	6.01	0.99	61.7	11.96	0.14	5.12	6.00	99.8
Mean ± SD							11.99 ± 0.18				
25 nM thioacetamide											
0	0.56	0.08	0.14	8.31	2.41	54.4	11.89	0.15	5.39	8.30	99.9
2	1.12	0.26	0.23	8.05	1.78	65.6	11.82	0.12	4.19	8.05	100
4	5.44	2.25	0.41	6.06	1.47	69.2	12.05	0.080	2.87	6.06	100
6	28.0	2.84	0.10	5.47	0.50	73.1	12.12	0.067	2.42	5.47	100
8	75.2	3.55	0.047	4.76		82.0	12.54	0.051	1.84	4.76	100
Mean ± SD							12.08 ± 0.28				
24 nM exudates											
0	0.56	0.08	0.14	6.28	1.00	58.0	11.98	0.072	2.57	6.28	100
2	5.44	0.49	0.090	5.79	0.61	59.8	12.68	0.063	2.27	5.79	100
4	23.2	0.50	0.022	5.78	0.43	76.6	12.28	0.048	1.73	5.78	100
6	58.5	0.79	0.014	5.49		90.0	11.96	0.038	0.38	5.49	100
8	96.1	1.01	0.011	5.27		104	12.27	0.032	1.13	5.27	100
Mean ± SD							12.23 ± 0.29				
2 μM SA											
0	0.56	0.08	0.14	8.38	2.41	33.8	12.01	0.19	6.8	6.52	77.9
2	1.14	1.86	1.63	6.52	2.32	39.1	11.70	0.13	4.5	5.30	81.3
4	5.80	2.30	0.40	6.08	2.17	48.6	12.32	0.09	3.4	5.16	84.9
6	24.5	2.76	0.11	5.62	0.98	49.9	12.08	0.08	3.0	4.80	85.3
8	71.4	2.96	0.041	5.42		55.1	12.42	0.07	2.7	4.70	86.7
Mean ± SD							12.10 ± 0.28				

would have been different from the characteristic sharp peak of sulfide and thiol compounds.

Comparison of copper complexation by synthetic thiols and ligands produced by the E. huxleyi cultures—Glutathione is known to complex copper as Cu⁺ in seawater (Leal and van den Berg 1998) and is detected as a ligand using the same titrations as used here. The stability of the copper complex with glutathione (log K'_{CuGS} = 10.9 ± 0.3) is similar to that of the complexes found in the *E. huxleyi* cultures (log K'_{CuL} = 10.3 ± 0.3). Thus, glutathione or a similar thiol-type ligand is a reasonable candidate for the unknown thiol-like ligands that give a reduction peak at -0.55 V.

For the thiols that displayed a reduction peak at -0.65 to -0.7 V (such as thioacetamide) to be good candidates for the identity of the copper-complexing ligands in the cultures, they have to form complexes with similar stability and be detected by the CSV titrations. These characteristics were investigated by determination of the complex formation of thioacetamide with copper in seawater. Thioacetamide (50 nM) was added to UV-irradiated seawater (free of other organic matter) and titrated with copper. The apparent C_L (experimentally measured) was 50.2 ± 3.5 nM, identical to the added thioacetamide concentration (which was therefore ful-

ly "recovered" by the titration), indicating 1:1 complex stoichiometry and a log K'_{Cu-thioacetamide} = 10.83 ± 0.08 (log K'_{Cu(II)-thioacetamide} = 12.39 ± 0.08, log K'_{Cu(I)-thioacetamide} = 15.98 ± 0.08). Thiol ligands of the thioacetamide type are therefore detected by the usual CSV titrations and form complexes similar in stability to those of copper with glutathione, which is known to form Cu⁺ complexes (Leal and van den Berg 1998) and with (perhaps slightly more stable) K'_{CuL} value similar to that of the ligands in the *E. huxleyi* cultures.

Effects of thiol compounds and exudates on E. huxleyi growth—We investigated whether the exudates in general, and thiol compounds specifically, could be of benefit to the algae. Therefore, *E. huxleyi* was incubated in parallel experiments in N/P-enriched seawater with and without either 25 nM thioacetamide (C_L = 54 nM), 24-nM exudates of a previous culture (filtrate of a 7-d control culture) (C_L = 58 nM), or 2 μM SA (because this ligand was used for the CSV measurements, it was not detected by this method; the C_L measured was identical to that of the control, 34 nM). SA was used because the adsorption of its complex with copper on the mercury drop electrode indicates that the complex is hydrophobic and possibly soluble in the cell membrane. The cultures were carried processed in duplicate, and the results

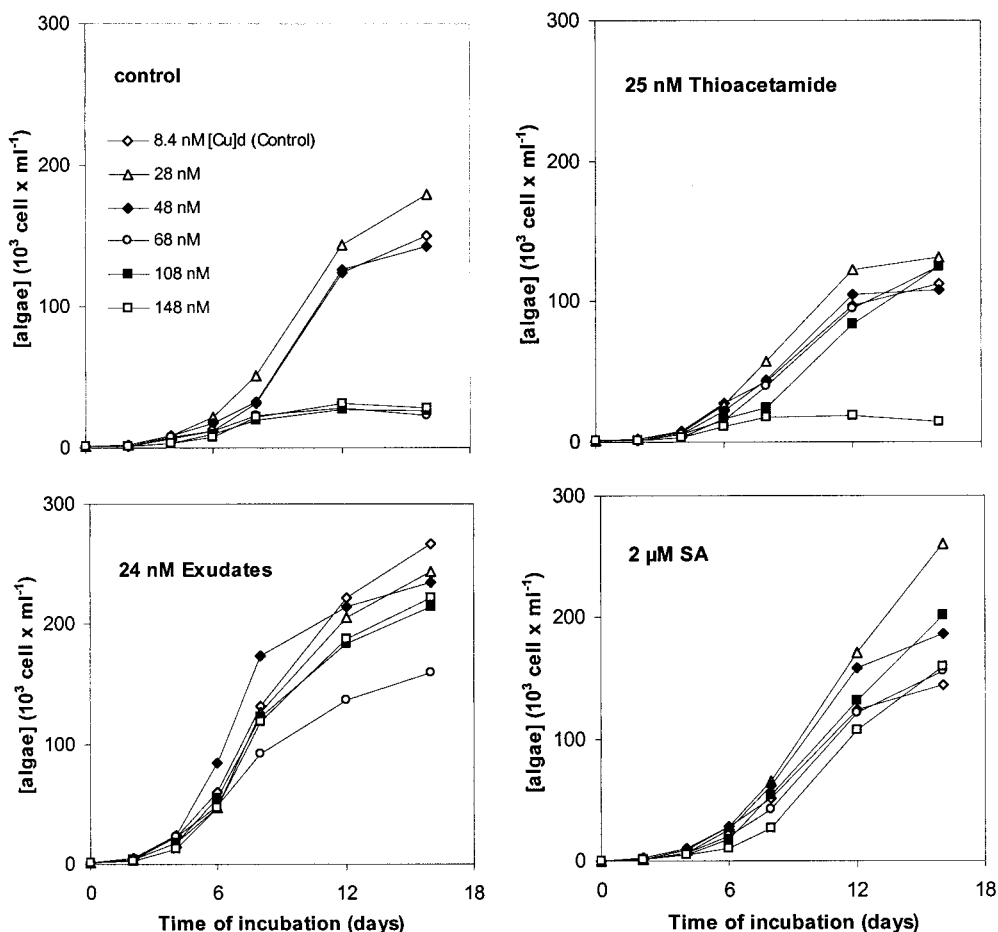


Fig. 5. Effect of adding organic ligands on the growth of *E. huxleyi* at various copper concentrations.

are presented in Table 3. The spread for the growth was <6% for the control and <25% for the other cultures. The growth rates reached their maximum after 8–12 d of growth (end of the exponential phase). The production of ligands, and the chemical speciation generally, was followed in detail over the first 8 d (Table 3).

Neither the added 25 nM thioacetamide nor the 2 μ M SA caused a significant change in the growth up to 8 d, whereas the 24-nM exudates caused a great increase in growth (Table 3). The exudates were clearly beneficial in some way. The uptake of copper was enhanced by the additions of thioacetamide and SA, whereas this uptake would have been lowered if these organic copper species were unavailable: $[\text{Cu}^{2+}]$ was lower than in the control culture, but growth was greater. In the earlier experiment (Table 2), growth was improved by increasing the copper concentration. This improved growth may not have been specific to increased $[\text{Cu}^{2+}]$ but to an increase in a different copper species, leading to improved copper uptake at low copper concentrations.

Table 3 shows that $[\text{Cu}]_{\text{algae}}$ was initially lowered by the addition of thioacetamide and the exudates, whereas it was initially increased by the addition of SA. The improved growth subsequently lowered the apparent $[\text{Cu}]_{\text{algae}}$ (by increasing the cell numbers) in all cultures (including that with

SA), thus masking an improved copper uptake on a per-cell basis. The $[\text{Cu}]_{\text{algae}}$ varied greatly over a range of 0.011–1.6 (maximum in the SA culture) fmol cell^{-1} , indicating that these algae can grow under a range of conditions. In view of their copper requirement (Table 2) and the reduced $[\text{Cu}]_{\text{algae}}$ when the cultures went into exponential growth, the initially high copper:cell ratios probably acted as copper storage, which was diluted during growth. Clearly uptake did not proceed at the same pace as the exponential algal growth, although sufficient copper was present in the solution.

Effect of copper additions to cultures with added ligands—In ligand-enhanced culture experiments at copper concentrations of 8.4 (no added copper), 28, 48, 68, 108, and 148 nM, the concentration at which copper toxicity occurred was increased (Fig. 5), indicating that the ligands buffered the copper availability until the copper concentration exceeded the ligand concentration. As before (Fig. 3), growth without ligand addition was suppressed at copper concentrations of 48 and 68 nM when the initial ligand concentration (34 nM) was exceeded. The toxic effect was not evident at a copper concentration of 48 nM even though this concentration exceeded the ligand concentration ($[\text{Cu}]_{\text{inorg}} = 14 \text{ nM}$, $[\text{Cu}^{2+}] = 0.40 \text{ nM}$), but C_L probably was greater

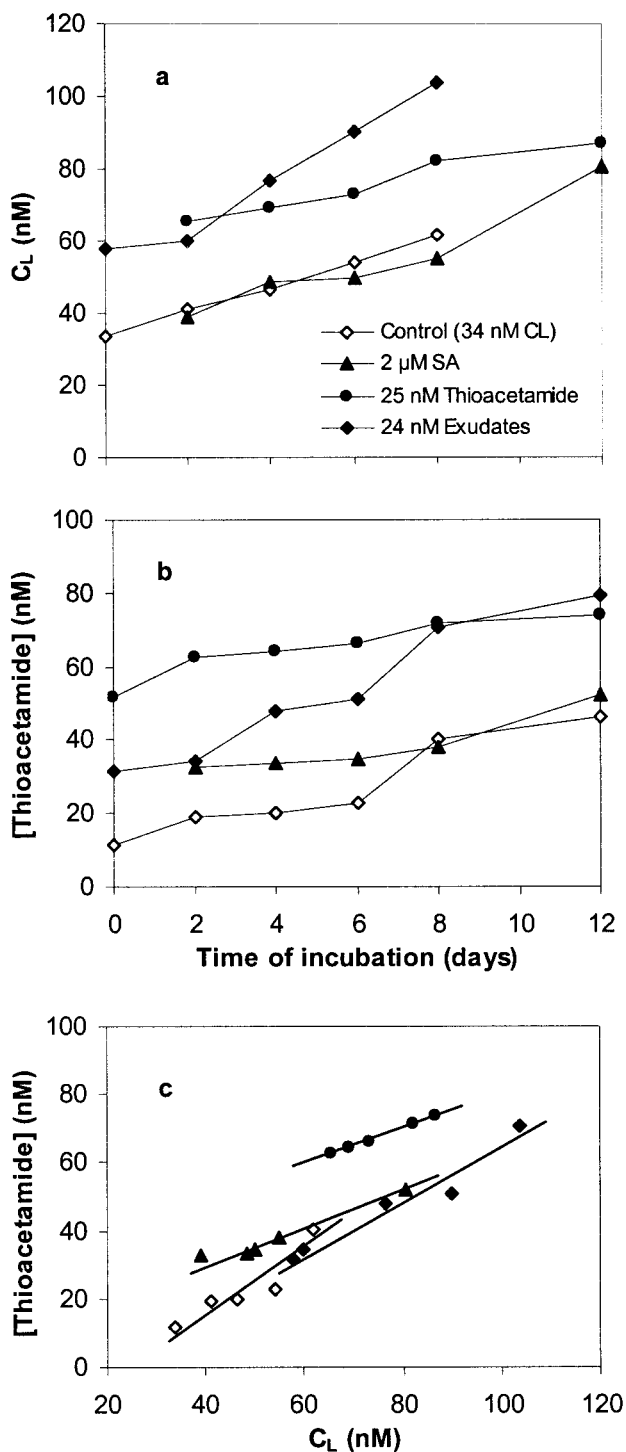


Fig. 6. *E. huxleyi* cultures supplemented with ligands and exudates. Production of complexing ligands (a) and of thiols (b) identified as thioacetamide as a function of the control culture age. Comparison of the concentrations of the produced thiol and complexing ligands (c).

than $[Cu]_d$ when exponential growth occurred after a lag time of ~ 8 d (the C_L in the control culture had increased to 65 nM during the same period).

Comparison with the cultures with added ligands shows

(Fig. 5) that growth was suppressed at much higher copper concentrations than before, when well in excess of the initial ligand concentration, indicating that copper toxicity was ameliorated by ligands produced during the incubations. For instance, in the culture to which 25 nM thioacetamide had been added ($C_L = 54$ nM), growth was suppressed only at the highest $[Cu]_d$ (148 nM); at 108 nM, the $[Cu]_d$ would have been a little more than the combined initial concentrations of ligands and thioacetamide (at $[Cu]_d = 108$ nM, 77% of the copper was organically complexed, $[Cu]_{inorg} = 25$ nM and $[Cu^{2+}] = 0.68$ nM), but the production of more ligands during the culture growth would have fully masked the added copper. However, the level of 148 nM $[Cu]_d$ ($[Cu]_{inorg} = 65$ nM) was apparently too high for *E. huxleyi*.

Ligand production in the cultures with added thiols—Measurements of the copper speciation in the cultures with the added thiols and SA (without added copper) showed that the ligand concentration increased as before with culture age (Table 3, Fig. 6). The thiol concentration also increased, paralleling the ligand concentrations in each culture (Fig. 6), with correlation coefficients ≥ 0.92 and with slopes near unity (0.90 and 0.78, respectively) for the control and the exudate-containing cultures; the slope was near 0.5 for the cultures with SA and thioacetamide (0.50 and 0.55, respectively). These results indicate that the thiol compounds constitute a major contributor of the exuded ligands and perhaps account for all of the ligands present in the seawater used for the cultures.

Although thioacetamide-like thiols were produced by all cultures, thiol production after 8 d of growth was less in the culture already containing 25 nM added thioacetamide (20 nM thioacetamide produced versus 29 nM in the control culture). However, the previous cultures showed that the production of thioacetamide-like thiols was stimulated by copper. It appears therefore that there is a feedback involving thiol production, the final thiol concentration, and the metal concentration in solution.

Production by algae as demonstrated here might help explain the widespread occurrence of thiols in seawater (Anderson et al. 1988; Matrai and Vetter 1988; Luther et al. 1991).

References

- AHNER, B. A., AND F. M. M. MOREL. 1995. Phytochelation production in marine algae. 2. Induction by various metals. *Limnol. Oceanogr.* **40**: 658–665.
- AHRLAND, S. 1988. Trace metal complexation by inorganic ligands in seawater, sect. 7. In T. S. West and H. W. Nurnberg [eds.], *The determination of trace metals in natural waters*. Blackwell Scientific Publications.
- AL-FARAWATI, R., AND C. M. G. VAN DEN BERG. 1997. The determination of sulfide in seawater by flow-analysis with voltammetric detection. *Mar. Chem.* **57**: 277–286.
- ANDERSON, L. G., D. DYRSSEN, AND P. O. J. HALL. 1988. On the sulphur chemistry of a super-anoxic fjord, Framvaren, South Norway. *Mar. Chem.* **23**: 283–293.
- BRAND, L. E., W. G. SUNDA, AND R. R. L. GUILLARD. 1983. Limitation of marine phytoplankton reproductive rates by zinc, manganese and iron. *Limnol. Oceanogr.* **28**: 1182–1198.

- , ———, AND ———. 1986. Reduction of marine phytoplankton reproduction rates by copper and cadmium. *J. Exp. Mar. Biol. Ecol.* **96**: 225–250.
- BRULAND, K. W., J. R. DONAT, AND D. A. HUTCHINS. 1991. Interactive influences of bioactive trace metals on biological production in oceanic waters. *Limnol. Oceanogr.* **36**: 1555–1577.
- BUCKLEY, P. J. M., AND C. M. G. VAN DEN BERG. 1986. Copper complexation profiles in the Atlantic Ocean. *Mar. Chem.* **19**: 281–296.
- CAMPOS, M. L. A. M., AND C. M. G. VAN DEN BERG. 1994. Determination of copper complexation in seawater by cathodic stripping voltammetry and ligand competition with salicylaldehyde. *Anal. Chim. Acta* **284**: 481–496.
- COALE, K. H., AND K. W. BRULAND. 1988. Copper complexation in the Northeast Pacific. *Limnol. Oceanogr.* **33**: 1084–1101.
- FLORENCE, T. M., H. K. J. POWELL, J. L. STAUBER, AND R. M. TOWN. 1992. Toxicity of lipid-soluble copper(II) complexes to the marine diatom *Nitzschia closterium*—amelioration by humic substances. *Water Res.* **26**: 1187–1193.
- GONZALEZ-DAVILA, M., J. M. SANTANA-CASIANO, J. PEREZ-PENA, AND F. J. MILLERO. 1995. Binding of Cu(II) to the surface and exudates of the alga *Dunaliella tertiolecta* in seawater. *Environ. Sci. Technol.* **29**: 289–301.
- IMBER, B. E., AND M. G. ROBINSON. 1983. Complexation of zinc by exudates of *Thalassiosira fluviatilis*. *Mar. Chem.* **14**: 31–41.
- KNAUER, K., R. BEHRA, AND L. SIGG. 1997. Effects of free Cu²⁺ and Zn²⁺ ions on growth and metal accumulation in freshwater algae. *Environ. Toxicol. Chem.* **16**: 220–229.
- LAGE, O. M., M. T. S. D. VASCONCELOS, H. M. V. M. SOARES, A. M. PARENTE, AND R. SALEMA. 1996. Suitability of the pH buffers DIPSO and HEPES for in vitro copper toxicity studies. *Arch. Environ. Contam. Toxicol.* **31**: 199–205.
- 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.
- LE GALL, A.-C., AND C. M. G. VAN DEN BERG. 1993. Cathodic stripping voltammetry of glutathione in natural waters. *Analyst* **118**: 1411–1415.
- , AND ———. 1998. Distribution of folic acid and glutathione in the water column of the North East Atlantic. *Deep-Sea Res. I*, **45**: 1903–1918.
- LUND, J. W. G., C. KIPLING, AND E. D. LE CREN. 1958. The inverted microscope method of estimating algal numbers and the statistical basis of estimations by counting. *Hydrobiologia* **11**: 144–170.
- LUTHER, G. W., III, T. M. CHURCH, AND D. POWELL. 1991. Sulfur speciation and sulfide oxidation in the water column of the Black Sea. *Deep-Sea Res.* **38**(suppl. 2A): S1121–S1137.
- MANPING, Z., G. BOSH, Z. SHENGBIN, AND L. LIANSHENG. 1990. Heavy metal complexation capacity of the South China Sea water. *Chin. J. Oceanol. Limnol.* **8**: 158–166.
- MATRAI, P. A., AND R. D. VETTER. 1988. Particulate thiols in coastal waters: The effects of light and nutrients on their planktonic production. *Limnol. Oceanogr.* **33**: 624–631.
- MOFFETT, J. W., AND L. E. BRAND. 1996. Production of strong, extracellular Cu chelators by marine cyanobacteria in response to Cu stress. *Limnol. Oceanogr.* **41**: 388–395.
- , ———, AND R. G. ZIKA. 1990. Distribution and potential sources and sinks of copper chelators in the Sargasso Sea. *Deep-Sea Res.* **37**: 27–36.
- MORELLI, E., AND G. SCARANO. 1994. Adsorptive stripping voltammetry of lumichrome in sea water at the static mercury drop electrode. *Anal. Chim. Acta* **296**: 277–284.
- PARSONS, T. R., Y. MAITA, AND C. M. LALLI. 1984. A manual of chemical and biological methods for seawater analysis. Pergamon.
- PLAVSIC, M., X. LU, AND C. M. G. VAN DEN BERG. 1997. Voltammetric study of the reactivity of folic acid in algal cultures and in natural waters. *Croat. Chem. Acta* **70**: 179–191.
- PRICE, N. M., G. I. HARRISON, J. G. HERING, R. J. HUDSON, P. M. NIREL, B. PALENIK, AND F. M. M. MOREL. 1989. Preparation and chemistry of the artificial algal culture medium Aquil. *Biol. Oceanogr.* **6**: 443–461.
- RUZIC, I. 1982. Theoretical aspects of the direct titration of natural waters and its information yield for trace metal speciation. *Anal. Chim. Acta* **140**: 99–113.
- SCARANO, G., AND E. MORELLI. 1996. Determination of phytochelators by cathodic stripping voltammetry in the presence of copper(II). *Anal. Chim. Acta* **319**: 13–18.
- STEIN, J. R. 1973. Handbook of physiological methods, culture methods and growth measurements, isolation and purification (section I). Cambridge University Press.
- , AND R. V. GESSNER. 1989. The production of extracellular copper-complexing ligands by marine and estuarine fungi. *Chem. Spec. Bioavail.* **1**: 65–70.
- SUNDA, W. G. 1995. The influence of nonliving organic matter on the availability and cycling of plant nutrients in seawater, p. 192–207. *In* R. G. Zepp and C. Sonntag [eds.], *Role of non-living organic matter in the earth's carbon cycle*. J. Wiley & Sons Ltd.
- VAN DEN BERG, C. M. G. 1982. Determination of copper complexation with natural organic ligands in seawater by equilibration with MnO₂. *I. Theory Mar. Chem.* **11**: 307–322.
- . 1984. Organic and inorganic speciation of copper in the Irish Sea. *Mar. Chem.* **14**: 201–212.
- , P. T. S. WONG, AND Y. K. CHAU. 1979. Measurement of complexing materials excreted from algae and their ability to ameliorate copper toxicity. *J. Fish. Res. Board Can.* **36**: 901–905.
- VAN DER WAL, P., R. S. KEMPERS, AND J. W. V. MARCEL. 1995. Production and downward flux of organic matter and calcite in a North Sea bloom of the coccolithophore *Emiliania huxleyi*. *Mar. Ecol. Prog. Ser.* **126**: 247–265.

Received: 30 July 1998

Accepted: 12 April 1999

Amended: 28 April 1999