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

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**Abstract**

Marine microalgae (*Emiliania 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 voltammetry. 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 salicylaldoxime (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 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$_3^-$, Cu(CO$_3$)$_2$$^{2-}$, CuOH$^-$, and Cu(OH)$_2^+$ 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-

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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 unsupplemented 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 NH₃ (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 salicylaldoxime (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 NH₃ was irradiated with ultraviolet (UV) light for 3 h to remove organic matter; 100 µl of this buffer in 10 ml seawater gave a pH of 8.35 (NBS pH scale).

Working standards of glutathione (Biochemicals), thiaoacetamide, 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 Bactotryptone, 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 µm 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-µm polycarbonate membrane, Millipore) using a peristaltic pump (Minipuls 3, Gilson). The filters, Erlenmeyer flasks, polycarbonate bottles, and plastics (pipette tips, filters, addition 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 nonselective 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. 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 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 x 10⁶ cells ml⁻¹). The synthetic seawater was prepared by dissolution of 23.38 g of...
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 through a 1-μm pore size filter (Sartorius) to avoid cell damage using a polycarbonate filter, Fisher Brand. The initial concentration of algae in the medium was approximately 5 × 10^6 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, 176 μM and 7.26 μM, respectively (equivalent to f/10 concentrations). The enriched seawater was sterilized by filtration through a 1-μm pore size filter (Sartorius) to avoid cell damage using a polycarbonate filter, Fisher Brand. The initial concentration of algae in the medium was approximately 5 × 10^6 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.

**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) to avoid cell damage using a polycarbonate (Sartorius) filter apparatus (0.4 μm polycarbonate membrane, Millipore, acid cleaned). Voltammetric equipment consisted of an Autolab voltammetric (Ecochemistry) connected to a Metrohm 663-V electrode stand (HMDE; drop size approximately 0.52 mm²).

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 voltammeteter was controlled by an IBM-AT compatible computer.

The [Cu]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 O₂-free nitrogen, using a 60-s adsorption step (tₐ) and a deposition potential (Eₐ) 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 [Cu]d was determined using a higher concentration of SA (50 μM) (giving a detection window centered at α = 10⁻⁵) and with Eₐ = −1.1 V, a large overpotential to include organically complexed copper in the analysis (thiol complexes dissociate under this condition; only complexes with K’Cuₐ > 10¹⁴ 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 NH₃ was used to approximately neutralize the pH prior to the reagent addition.

Copper in the algae ([Cu] 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 (Cₐ) and K’Caₐ 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 [Cu]labile had been measured. The volt-amperometric 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.
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 $E = 0.58$ 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 $[\text{Cu}]_{\text{labile}}$ in the titrations is defined by the copper, which equilibrates with and is bound by the added SA. $[\text{Cu}]_{\text{labile}}$ 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:

$$[\text{Cu}]_{\text{labile}} = [\text{Cu}]_{\text{SA}} + [\text{Cu}]_{\text{org}} = (\alpha_{\text{CuSA}} + \alpha_{\text{CuII}})[\text{Cu}^{2+}].$$

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):

$$[\text{Cu}]_{\text{labile}}/[\text{Cu}(\text{II})L] = [\text{Cu}]_{\text{labile}}/C_L + (\alpha_{\text{Cu(II)}} + \alpha_{\text{CuSA}})/(C_L K_{\text{Cu(II)L}}).$$

where the conditional stability constant $K'_{\text{Cu(II)L}} = [\text{Cu}(\text{II})L]/[\text{Cu}^+][L]$; $\alpha_{\text{Cu(II)}}$ is the $\alpha$ 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 $\alpha_{\text{CuSA}}$ is the $\alpha$ coefficient for complexation of copper(II) by SA:

$$\alpha_{\text{CuSA}} = K'_{\text{CuSA}}[\text{SA}'] + \beta'_{\text{CuSA}2}[\text{SA}]^2.$$ (Campos and van den Berg 1994). $C_L$ was calculated from the slope of the linear least-squares regression, whereas $K_{\text{Cu(II)L}}$ 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

$$K_{\text{Cu(L')}} = [\text{CuL}]/([\text{Cu}'][L']).$$

where $[\text{Cu}']$ is the concentration of copper not complexed by L. In this constant the oxidation state of copper is left
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 $\geq 0.99$) 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 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 $K'_\text{Cu(II)}$, as if copper occurs as copper(I). A value for $K'_\text{Cu(II)}$ is calculated from $K_\text{Cu(II)}$ using the following relationship:

$$K'_\text{Cu(II)} = \frac{K_\text{Cu(II)}}{a_{\text{Cu(II)}}}.$$  

(5)

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

$$K'_\text{Cu(I)} = K'_\text{Cu(II)} \times a_{\text{Cu(I)}},$$  

(6)

where $a_{\text{Cu(I)}}$ is the $a$ 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$_2$O$_2$; Cu(I)$_{\text{inorg}}$ ~ 0.1 M). The inorganic copper concentration was then calculated using

$$[\text{Cu}^{\text{II}}]_{\text{inorg}} = \{ -b + (2b - 4ac)^{1/2} \} / 2a$$  

(7)

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

$$[\text{Cu}^{\text{II}}]_{\text{org}} = [\text{Cu}^{\text{II}}]_{\text{Cu(II)}}.$$  

(8)

and the organic copper concentration was calculated using

$$[\text{Cu}^{\text{II}}]_{\text{org}} = ([\text{Cu(II)L}] - [\text{Cu}^{\text{II}}]_{\text{Cu(II)}} - [\text{Cu}^{\text{II}}]_{\text{org}}.$$  

(9)

Results

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

<table>
<thead>
<tr>
<th>Culture age (d)</th>
<th>Cell number (10$^3$ cell ml$^{-1}$) Algae (pmol cell$^{-1}$) Filtrate</th>
<th>N (μM)</th>
<th>P (μM)</th>
<th>N/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0*</td>
<td></td>
<td>176</td>
<td>7.26</td>
<td>24</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>174</td>
<td>7.61</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>134</td>
<td>2.99</td>
<td>8.7</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>101</td>
<td>0.18</td>
<td>9.8</td>
</tr>
</tbody>
</table>

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$^{\text{II}}_c$]) on the growth (b) and growth rates (c) of the cultures.
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 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 HEPPS 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 × 10⁹ 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 control 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

### 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.

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>[Algae] (10^10 cell ml⁻¹)</th>
<th>[Cu]_{algae} (pM)</th>
<th>[Cu]_{algae} (nM)</th>
<th>[Cu]_{labile} (nM)</th>
<th>C_{i} (nM)</th>
<th>Log K_{Cu labile}</th>
<th>[Cu^{2+} ] (pM)</th>
<th>[Cu]_{inorg} (pM)</th>
<th>[Cu]_{org} (nM)</th>
<th>CuL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.28</td>
<td>0.04</td>
<td>0.14</td>
<td>8.36</td>
<td>6.55</td>
<td>34.2</td>
<td>12.10</td>
<td>0.43</td>
<td>15.3</td>
<td>8.34</td>
</tr>
<tr>
<td>2</td>
<td>0.65</td>
<td>0.38</td>
<td>0.58</td>
<td>7.98</td>
<td>4.63</td>
<td>43.7</td>
<td>11.86</td>
<td>0.29</td>
<td>10.6</td>
<td>7.97</td>
</tr>
<tr>
<td>7</td>
<td>8.07</td>
<td>1.74</td>
<td>2.22</td>
<td>6.62</td>
<td>4.19</td>
<td>58.6</td>
<td>12.13</td>
<td>0.17</td>
<td>6.04</td>
<td>6.61</td>
</tr>
<tr>
<td>10</td>
<td>25.4</td>
<td>2.09</td>
<td>0.08</td>
<td>6.27</td>
<td>3.37</td>
<td>68.7</td>
<td>11.72</td>
<td>0.13</td>
<td>4.76</td>
<td>6.27</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>8.4 ± 0.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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Copper, thios, and Emiliania

1755
A 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 (<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
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]_{algae}) was evaluated from [Cu]_{algae} 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\(^{-1}\) (5–250 × 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]_{algae} 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]_{algae} = 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-
The concentration of each individual thiol (such as glutathione or thioacetamide) was $\sim 0.5$ nM, and the combined concentrations of glutathione and thiols were similar to $C_t$. 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 $C_t$ was not identical to the thiol concentrations. The similar magnitude and covariance 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 phytochelatins (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.

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<th>[Cu]_{labile} (nM)</th>
<th>C_L (nM)</th>
<th>Log K_{CuL}</th>
<th>[Cu^{2+}] (pM)</th>
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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 $K'_{CuGSH} = 10.9 \pm 0.3$) is similar to that of the complexes found in the *E. huxleyi* cultures (log $K'_{CuL} = 10.3 \pm 0.3$). 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 thiol 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 $50.2 \pm 3.5$ nM, identical to the added thioacetamide concentration (which was therefore fully “recovered” by the titration), indicating 1:1 complex stoichiometry and a log $K'_{Cu-thioacetamide} = 10.83 \pm 0.08$ (log $K'_{CuDMSO-thioacetamide} = 12.39 \pm 0.08$, log $K'_{CuDMSO-thioacetamide} = 15.98 \pm 0.08$). 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 NP-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
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 µM 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: [Cu(II)] 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 [Cu(II)] but to an increase in a different copper species, leading to improved copper uptake at low copper concentrations.

Table 3 shows that [Cu]_{algal} 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 [Cu]_{algal} (by increasing the cell numbers) in all cultures (including that with SA), thus masking an improved copper uptake on a per-cell basis. The [Cu]_{algal} 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 [Cu]_{algal} 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 ([Cu]_{inorg} = 14 nM, [Cu(II)] = 0.40 nM), but C_L probably was greater.

![Graphs showing Effect of adding organic ligands on the growth of *E. huxleyi* at various copper concentrations.](image-url)
than [Cu] 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]$_L$ (148 nM); at 108 nM, the [Cu]$_L$ would have been a little more than the combined initial concentrations of ligands and thioacetamide (at [Cu]$_L = 108$ nM, 77% of the copper was organically complexed, [Cu]$_{org} = 25$ 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]$_L$ ([Cu]$_{org} = 65$ 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**


Leal et al.


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