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Release of weak and strong copper-complexing agents by algae[1]

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Abstract

By means of potentiometric titrations, the copper-complexing properties of organic compounds released by 21 algal species are characterized. Most eucaryotic species released measurable amounts (10^{-6} M) of copper-complexing agents that could be modeled as weak organic acids with $\log\{{}^{*}K_{CuL}\} = 0.5$. Of seven blue-green algal species studied, four produced about 5×10^{-7} M concentrations of strong copper-complexing agents with conditional stability constants in the range 10^{8} to 10^{12}; two produced complexing agents similar to those produced by the eucaryotes. In time-course experiments with three chlorophytes and two cyanophytes, the release of measurable concentrations of copper-complexing agents occurred principally during stationary phase.

Trace metals can control algal growth by acting either as toxicants (e.g. copper), or as limiting micronutrients (e.g. iron). Toxicity of and nutrient limitation by trace metals depend on their chemical speciation in the medium (Sunda and Guillard 1976; Anderson and Morel 1978; Anderson et al. 1978). In turn, the speciation of metals in natural waters and in culture media is controlled by chemical processes such as precipitation, adsorption, and inorganic and organic complexation (Stumm and Morgan 1970; Morel et al. 1979*a*). By releasing metabolites that complex metals, algae could, in principle, modify metal speciation in the medium and effectively control metal availability or toxicity in their external milieu.

To have a significant effect on the trace metal chemistry of natural waters, an organic ligand must have relatively high affinity for metals; greater, for example, than that of simple amino acids and organic acids (Stumm and Morgan 1970; Stumm and Brauner 1975; Morel et al. 1974; Jackson and Morgan 1978). Hydroxamic acids may be the only examples of strong metal-complexing agents that are known to be released by algae and to be present in natural waters (Anderegg et al. 1963; Neilands 1967, 1973, 1974; Simpson and Neilands 1976; Murphy et al. 1976). Simpson and Neilands (1976) identified a polyhydroxamate siderochrome, called schizokinen, produced by *Anabaena* sp. and Murphy et al. (1976) found bound hydroxamates in cultures of *Anabaena flos-aquae* and in field samples from blooms of *Anabaena* sp. The role of siderochromes in microbial iron transport has been established from studies of iron mutants of *Salmonella typhimurium* (Luckey et al. 1972) and *Bacillus megaterium* (Davis and Byers 1971) and presumably a similar transport mechanism exists in blue-green algae.

Fogg and Westlake (1955) demonstrated complexation and detoxification of copper by extracellular polypeptides from cultures of *Anabaena cylindrica*. They found that the pH titration of a copper sulfate-extracellular polypeptide solution was depressed relative to the sum of separate pH titrations of copper and polypeptide solutions and that the addition of extracellular polypeptide decreased the toxicity of copper to cultures of *A. cylindrica*. Since then, researchers have elucidated the importance of organic complexation in determining copper toxicity to algae. Using synthetic chelators whose copper-complexing properties are known, several researchers have shown that the toxicity of copper to several marine algae is a unique function of the cupric ion activity (Sunda and Guillard 1976; Anderson and Morel 1978).

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The other major result of the work of Fogg and Westlake—the demonstration of copper-complexing agents release by the algae themselves—has been cited frequently and used to explain experimental results (Johnston 1964; Barber et al. 1971; Barber 1973; Steemann Nielsen and Wium-Anderson 1971; Lange 1974; Huntsman and Barber 1975). However, there has been little confirmation of this result in the recent literature. For example, in a screening of eight algal species only one, *Gloeocystis gigas*, was found to produce compounds that reduced significantly the cupric ion activity at 10^{-6} M total copper (Swallow et al. 1978).

The purpose of this study was to investigate if the generally negative results of Swallow et al. (1978) extend to lower exudate concentrations (10^{-7} M) and to other algal species, especially cyanophytes. Twenty-one species were screened for production of copper-complexing agents by examining samples from the stationary phase of growth and several species were studied during exponential growth and stationary phase.

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Methods

Culture of algae and sample preparation—The algae were grown in either medium WC (Guillard 1975) or blue-green algal medium (Allen 1968); in both cases the trace metal recipe followed that of Aquil (Morel et al. 1979*b*) without EDTA. The media were sterilized by autoclaving, Allen medium was autoclaved 2 weeks before inoculation to permit redissolution of the precipitate. To obviate the difficulties usually encountered in culture media devoid of chelating agents, we added ferric chloride immediately before inoculation as in Swallow et al. (1978).

The species studied and their culture conditions are listed in Table 1. Sterile techniques were used throughout. Most cultures were grown in 250 ml of medium in 500-ml Erlenmeyer flasks at constant temperature (20°C) and in continuous light (20 μeinst$\cdot$cm$^{-2}\cdot$s^{-1}). The WHOI dinoflagellates were grown at 15°C and under a light/dark cycle of 14:10 h. In the time-course experiments the cultures were grown in 500 ml of medium in 1,000-ml Erlenmeyer flasks. Growth of cultures was followed by counts with a hemocytometer or by measurements of chlorophyll concentrations (Strickland and Parsons 1972). Filtrates from algal cultures 3 days into stationary phase were prepared following Swallow et al. (1978). In the time-course experiments, samples were taken at 3- or 4-day intervals.

Titrations—The complexing properties of algal exudates were characterized by performing two types of titrations with a cupric ion electrode: one in which total copper is increased at constant pH and the other in which pH is varied at constant total copper. The basic electrode apparatus and titration procedures were the same as used by Swallow et al. (1978). However, to permit measurement of 10^{-7} M equivalent ligand concentration, we improved the accuracy and sensitivity of the original method by making the following modifications:

In the copper titrations, copper nitrate was added incrementally, starting at a concentration of 10^{-7} M and continuing to 10^{-4} M and in the pH titrations 6×10^{-7} M copper nitrate was added.

The pH was buffered at 6.25 ± 0.05 during the copper titrations by adjusting the alkalinity of the sample and bubbling with 1% CO_2(g) balanced with N_2(g).

We chose pH and P_{CO_2} for the copper titrations to optimize for the complexation of copper by weak organic acids obeying the reaction

$$Cu^{2+} + HL \rightleftarrows CuL^{+} + H^{+}, \qquad {}^{*}K_{CuL}, \quad (1)$$

and to avoid the formation of copper hydrolysis species and minimize the proportion of copper bound in $CuCO_3$(aq).

Table 1. Algal species and experimental culturing conditions.

	Medium	Growth rate k (div·d^{-1})	Conditions of stationary phase: cells·ml^{-1}	Conditions of stationary phase: mg Chl a·liter^{-1}
	Chlorophyta			
Chlorella pyrenoidosa UTEX 1663	WC	1.7	2×10^6	1.5
Oocystis polymorpha UTEX 1645	WC	2.5	3×10^5	0.5
Pandorina morum UTEX 1725	WC	2.3	1×10^5	1.5
Gloeocystis gigas UTEX 291	WC	2.0	2×10^5	0.7
Chlamydomonas sp. G6 WHOI	WC	3.2	3×10^5	2.0
Chlamydomonas sp.* 6/33 WHOI	WC		3×10^5	
*Chlorella autotrophica** UTEX 580	WC		1×10^6	
Scenedesmus obliquus UTEX 1592	WC	2.2	1×10^6	
	Bacillariophyta			
Nitzschia palea UTEX 1813	WC	2.2	1×10^6	
*Thalassiosira pseudonana** (3H) WHOI	WC		1×10^6	
*Thalassiosira weissflogii**† (Actin) WHOI	WC		1×10^5	
	Chrysophyta			
Tribonema aequale UTEX 50	WC		5×10^5	
Synura petersenii WHOI	WC	1.0	2×10^5	
	Pyrrophyta			
Freshwater dinoflagellates WHOI	WC	0.4	2×10^4	
	Cyanophyta			
Anabaena cylindrica UTEX B1611	WC	1.2		5.0
Anabaena flos-aquae UTEX 1444	WC	1.2		1.5
Nostoc muscorum UTEX 1832	Allen			
Gloeocapsa alpicola 6308L	Allen	1.6		4.0
Microcystis aeruginosa UTEX 2061	Allen	0.7		1.0
Anacystis nidulans 6301L	Allen	1.7		3.0
Coccomyxa chodatii UTEX 279	Allen	0.4		0.3

* Marine or estuarine algal species.
† Grown in WC medium with 1/10 SOW added.

The speciation of copper in copper titrations of phosphate-free WC medium was computed. The computer program MINEQL (Westall et al. 1976) was used to make all chemical equilibrium calculations reported here. Malachite or tenorite are predicted to precipitate at copper concentrations $>5 \times 10^{-6}$ M. However, in the region 10^{-6} to 10^{-4} M total copper during the titrations of medium and salt blanks the electrode response followed $(Cu^{2+}) = Cu_T$ (McKnight 1978). Also, the electrode response at the final copper concentration (10^{-4} M) did not indicate a significant decrease in cupric ion activity even after 24 h. The kinetics of malachite or tenorite precipitation at pH 6.25 are assumed to be slow and these solids are not included when we compare computed copper titrations to experimental results. When precipitation is ignored, most of the copper in a copper titration of phosphate-free WC medium is calculated to be Cu^{2+} (83%), with the rest found in $CuCO_3$(aq) (13%), $CuSO_4$(aq) (2.3%), and $CuOH^+$(1.8%).

The limit of detection of the method for weak ligands is determined by loss of copper from solution (presumably to the glass beaker) at low total copper concentrations (10^{-7} to 10^{-6} M) in chelator-free medium (McKnight 1978). For copper titrations of algal culture media to be unambiguously interpreted as demonstrating the production of extracellular copper-complexing compounds, the depression of the cupric ion activity must be at least equal to that for 10^{-6} M of a weak ligand with $\log \{^*K_{CuL}\} = 0.5$.

All the data have been converted from

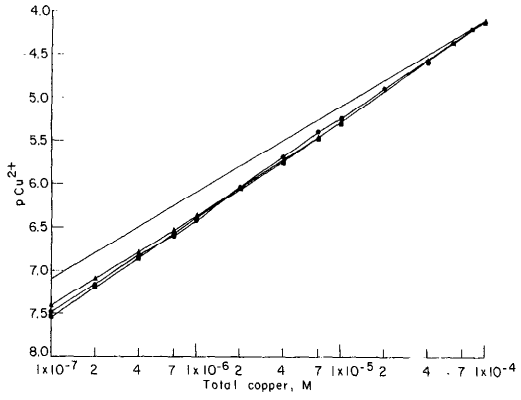

Fig. 1. Experimental copper titrations of background electrolyte with 1×10^{-5} M glycine (● and ■) and calculated copper titrations of WC medium (——) and background electrolyte (10^{-3} M KNO_3) with 1×10^{-5} M glycine (▲).

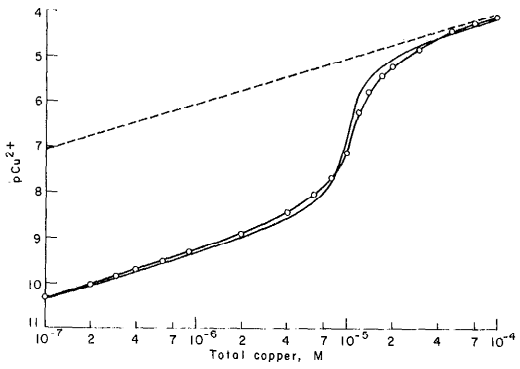

Fig. 2. Experimental copper titration of background electrolyte with 1×10^{-5} M Desferal (○), a trihydroxamate siderochrome from CIBA-GEIGY, and calculated copper titrations of WC medium (– – –) and background electrolyte (10^{-3} M KNO_3) with 1×10^{-5} M of strong ligand with $\log\{^cK\} = 8.4$ (——).

millivolts (E) to $p\mathrm{Cu}^{2+}$ ($= -\log\{\mathrm{Cu}^{2+}\}$) by means of the Nernst equation

$$E = E^0 - \alpha \cdot p\mathrm{Cu}^{2+}$$

in which the electrode slope α, was taken between 30 and 27.5 mV based on measurements made before each titration of electrode potential in 10^{-7}, 10^{-6}, and 10^{-5} M copper solutions. The calculation of (E^0) is described by McKnight (1978).

Copper titrations of solutions of glycine and Desferal, a trihydroxamate siderochrome (CIBA-GEIGY), were compared to calculations. Both compounds are representative of organic complexing agents that algae may be expected to release. For 10^{-5} M glycine the experimental titrations and calculations from published formation constants are in good agreement (Fig. 1), even in the range 10^{-7} M to 10^{-6} M total copper. This agrees with the results of Vuceta (1976) that adsorption of copper on SiO_2(s) is not significant if the copper is complexed by a competing ligand. Figure 2 shows the copper titration of a 10^{-5} M solution of Desferal and a fitted copper titration with $\log\{^cK\} = 8.4$ and total ligand concentration (L_T) = 10^{-5} M for the reaction

$$\mathrm{Cu}^{2+} + \mathrm{L}^- = \mathrm{CuL}^+, \qquad {}^cK. \qquad (2)$$

The response of the electrode is linear at the low cupric ion activities ($10^{-10.3}$ to 10^{-9}) below the equivalence point, which supports the assumption of Nernstian behavior. The formation constant from the fitted titration, $\log\{^cK\} = 8.4$, is slightly higher than the formation constants for copper-hydroxamate complexes reported by Anderegg et al. (1963), $\log\{^cK\} = 7.9$ and 7.6.

Chemical analysis—Bound hydroxamate was measured by the Csáky (1949) method with minor modifications. As in Strickland and Parsons (1972), N-1-naphthylenediamine (1.35 g N-1-N dihydrochloride in 250 ml 30% acetic acid) was used to form the azo dye in the final nitrite determination.

Results

The results of Swallow et al. (1978) suggest that concentrations of copper-complexing agents greater than micromolar are not released in most cultures of eucaryotic algae. We did the following experiments to see if this is true for lower concentrations of copper-complexing agents and blue-green algae.

Eucaryotic algae—Figures 3 and 4 show the copper and pH titrations of filtrates from a stationary phase culture of *Chlorella autotrophica* and the calculated

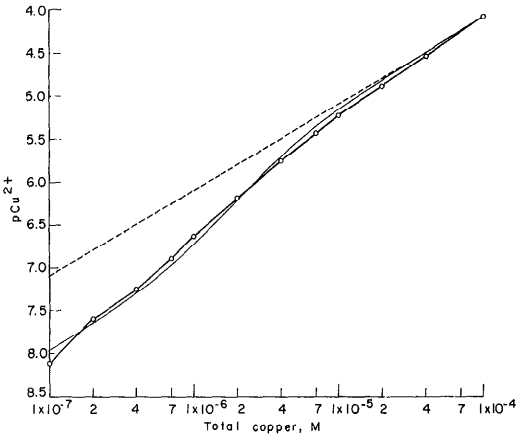

Fig. 3. Experimental copper titration of *Chlorella* medium (O) and calculated copper titrations of WC medium (– – –) and WC medium with 1.5×10^{-6} M of weak ligand ($\log\{{}^*K_{CuL}\} = 0.5$) (——).

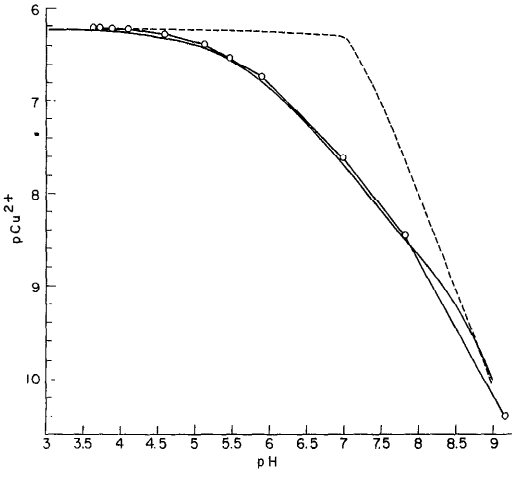

Fig. 4. Experimental pH titration of *Chlorella* culture medium (O) and calculated pH titration of WC medium (– – –) and WC medium with 1.5×10^{-6} M weak ligand ($\log\{{}^*K_{CuL}\} = 0.5$) (——), for $\log\{{}^*\beta_2\} = 17.3$. Copper–algal complex calculated to be major copper species from pH 5.5 to 8.5, regardless of which value of ${}^*\beta_2$ is used for $Cu(OH)_2(aq)$.

copper and pH titrations for $L_T = 1.5 \times 10^{-6}$ M and $\log\{{}^*K_{CuL}\} = 0.5$ and for medium blanks. The cupric ion activities are significantly lower in *Chorella* culture medium than in medium blanks in the appropriate regions of both copper and pH titrations (10^{-7} to 7×10^{-6} M total copper, and pH 5 to pH 8). Furthermore, the same values of ${}^*K_{CuL}$ and L_T fit both copper and pH titrations, which supports the original assumption of a weak organic acid that obeys reaction 1. These titrations of *Chlorella* culture medium are representative of titrations for other eucaryotic algae that produced measurable concentrations of copper-complexing agents.

The results for all eucaryotic algae studied are presented in Table 2 in terms of equivalent ligand concentrations (L_T) and formation constants (${}^*K_{CuL}$) that give a reasonable fit to the experimental copper and pH titrations. The five chlorophytes and two chrysophytes produced measurable concentrations of copper-complexing agents, evidenced by values for L_T (Table 2) $>1 \times 10^{-6}$ M. As before (Swallow et al. 1978), *G. gigas* produced 3×10^{-6} M complexing agent, more than any of the other eucaryotic algae studied. The copper titrations for the two estuarine diatoms, grown in freshwater medium, are close to the limit of detection of the method for weak ligands. The values for L_T and ${}^*K_{CuL}$ were first estimated graphically and then improved by trial and error from comparison of calculated titrations with experimental titrations.

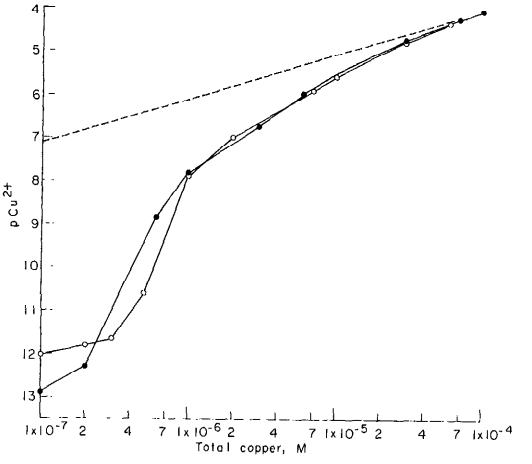

Fig. 5. Experimental copper titrations of *Microcystis aeruginosa* (●) and *Anacystis nidulans* (O) culture medium, and calculated copper titration of Allen medium (– – –).

Table 2. Production of copper-complexing agents by eucaryotic algae (copper and pH titrations are given in McKnight 1978).

	No. Cu titrations	No. pH titrations	L_T(M)	$\log\{^*K_{CuL}\}$
		Chlorophyta		
Oocystis polymorpha	1	–	—	—
Scenedesmus obliquus	1	–	—	—
Chlorella pyrenoidosa	1	–	—	—
Pandorina morum	3	1	1.5×10^{-6}	0.5
Gloeocystis gigas	2	–	3×10^{-6}	0.5
Chlamydomonas sp. G6	1	1	1.5×10^{-6}	0.5
Chlorella autotrophica	1	1	1.5×10^{-6}	0.5
Chlamydomonas sp. 6/33	1	1	1.5×10^{-6}	0.5
		Bacillariophyta		
Nitzschia palea	1	–	—	—
Thalassiosira pseudonana	1	–	1×10^{-6}	0.5
Thalassiosira weissflogii	1	–	1×10^{-6}	0.5
		Chrysophyta		
Tribonema aequale	1	–	1.5×10^{-6}	0.5
Synura petersenii	2	–	1.5×10^{-6}	0.5
		Pyrrophyta		
Freshwater dinoflagellates	1		—	—

Since the titrations of culture medium from the eucaryotic algae producing complexing agents were similar, only one value of $\log\{^*K_{CuL}\}$ was used (0.5), although for any one titration a $\log\{^*K_{CuL}\}$ of 0.6 or 0.4 might also have given a good fit. The range in equivalent ligand concentration (L_T) which would fit any one titration was also small, $1 \pm 0.2 \times 10^{-6}$ M. From copper titrations of media from different cultures of both *Pandorina morum* and *Synura petersenii*, the variability between cultures was $<5 \times 10^{-7}$ M or 50% of L_T. Since the variability between cultures of the same species was greater than the range in possible values of L_T to fit any one titration, we did not seek a method for calculating a statistical best fit to a titration.

The copper titrations of media from cultures of the five other freshwater eucaryotes are generally not distinguishable from the medium blanks and show that these species do not produce significant concentrations of copper-complexing agents under these culture conditions. The exceptions are the results for *Scenedesmus obliquus* and *Oocystis polymorpha* at copper concentrations $>4 \times 10^{-6}$ M which can be interpreted as representing 10^{-5} M concentrations of a complexing agent similar in its copper-complexing properties to glycine ($\log\{^*K_{CuL}\} = -1.2$) (Sillén and Martell 1964, 1971).

Procaryotic algae—From the studies of the production of hydroxamate siderochromes, we know that cyanophytes can change the speciation of iron in natural waters. The following experiments were done to determine whether cyanophytes can change the speciation of copper as well.

The copper titrations of media from cultures of *Microcystis aeruginosa* and *Anacystis nidulans* are shown in Fig. 5 and the results for all seven blue-green algae studied are presented in Table 3. Those for five species are presented only in terms of the measured cupric ion activity at 10^{-7} M total copper because the copper titrations could not be fitted by a simple, single ligand, complexation reaction. For four cyanophytes, *A. flosaquae*, *A. nidulans*, *M. aeruginosa*, and *Gloeocapsa alpicola*, these cupric ion activities are much lower ($10^{-9.5}$ to $10^{-14.5}$) than in any of the titrations of eucaryotic

Table 3. Production of copper-complexing agents by cyanophytes (copper titrations are given in McKnight 1978).

Cyanophyta	No. Cu titrations	L_T	$^*K_{CuL}$	$p\{Cu^{2+}\}$ at $Cu_T = 10^{-7}$M	Concn bound hydroxamate (M)
Anabaena flos-aquae	3			9.5	3.7×10^{-6}
Gloeocapsa alpicola	3			10.8, 13.2, 14.5	1.5×10^{-5}
Anacystis nidulans	1			12.0	6.7×10^{-5}
Microcystis aeruginosa	1			12.9	1.6×10^{-5}
Coccomyxa chodatii	1			8.5	2.9×10^{-5}
Anabaena cylindrica	1	2×10^{-6}	$10^{0.5}$	8.2	3.4×10^{-6}
Nostoc muscorum	1	5×10^{-6}	$10^{0.5}$	8.1	3.0×10^{-5}

algal media, showing that these cyanophytes produce much stronger copper-complexing agents. Although equilibration occurs within a few hours and the potential increases significantly with additions of 10^{-7} M copper, we assume Nernstian response at these low potentials with reservations. Since a precipitate was observed at high copper concentrations in *G. alpicola* and *Coccomyxa chodatii* media, for these two species the conversion of the measured potentials to cupric ion activities is based on measurements in copper standards before and after titrations, and titrations do not intersect $(Cu^{2+}) = Cu_T$. The copper titrations of media from different cultures of *A. flos-aquae* are reproducible; those of media from different cultures of *G. alpicola* vary considerably, probably because the samples were not taken at exactly the same time in stationary phase.

Not all the cyanophytes studied excreted measurable concentrations of strong copper-complexing agents. The results for *A. cylindrica* and *Nostoc muscorum* show the presence of greater than micromolar concentrations of weak ligands, obeying reaction 2, and similar to those from eucaryotic algae. In the copper titration of culture medium from *C. chodatii* there is an inflection in the cupric ion activity at 5×10^{-7} M total copper and precipitation at higher concentrations.

The copper titrations of media from stationary phase cultures of *A. nidulans* and *M. aeruginosa* (Fig. 5) illustrate the other major difference between the copper-complexing agents produced by eucaryotic and blue-green algae. The copper titrations of cyanophycean culture media cannot be modeled with weak organic acids that obey the complexation reaction 1 over the complete range of the titrations. The pH titrations of *A. flos-aquae* and *G. alpicola* culture media are also inconsistent with the weak organic acid assumption, since at pH 3 the cupric ion activities are depressed by an order of magnitude. Above neutrality, the equilibration is exceedingly slow and the back titration is inconsistent with the forward titration. Furthermore, the copper titrations cannot be fitted with one ligand obeying the simpler complexation reaction 2. However, by assuming the presence of two or more ligands and varying their concentrations and formation constants, we can find many ways to fit the titrations. For example, from the copper titrations of *A. nidulans* and *M. aeruginosa* we see that these coccoid cyanophytes produce at least 8×10^{-7} and 5×10^{-7} M of strong copper-complexing agents, with effective formation constants of about 10^{11} and 10^{12}, at pH 6.3.

Four of the blue-green algal species studied produce copper-complexing agents that are much stronger than any produced by the eucaryotic algal species grown under similar conditions. An obvious question is whether the production of strong copper-complexing agents cor-

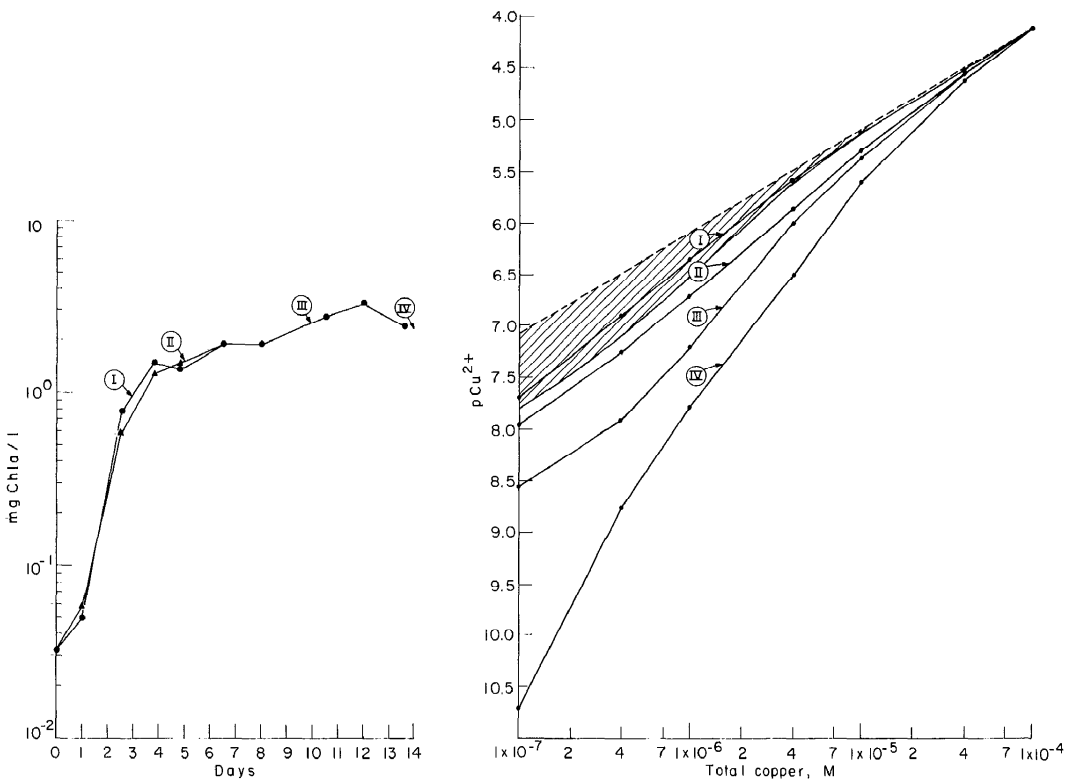

Fig. 6. Time-course experiment with *Chlamydomas* sp. Left—growth curves for duplicate cultures. Right—experimental copper titrations of culture medium samples taken at indicated time on growth curves. Hatched area bounded by calculated copper titration of WC medium and calculated copper titration of WC medium with 1×10^{-6} M of weak ligand ($\log\{{}^{*}K_{CuL}\} = 0.5$) and represents limits of detection of method for weak ligands (*see text*).

relates with the production of iron-complexing siderochromes by cyanophytes. Analysis for bound hydroxamates provides an indication of the presence of hydroxamate siderochromes (Simpson and Neilands 1976; Murphy et al. 1976). We analyzed samples from the stationary phase of all the algal species for bound hydroxamates and found, as did Murphy et al. (1976), no detectable bound hydroxamate in samples from any of the eucaryotic algal cultures. Bound hydroxamate was present in all seven blue-green algal cultures in concentrations ranging from 7×10^{-5} to 3×10^{-6} M (Table 3). There is, however, no correlation among high or low concentrations of bound hydroxamate and detection of strong copper-complexing agents. We confirmed that the bound hydroxamate in *A. nidulans* culture medium is a trihydroxamate siderochrome by showing that the yellow absorbance (440 nm) was maintained down to pH 2.75, while the purple absorbance (510 nm) that is characteristic of monohydroxamic acid never developed (Neilands 1967).

Release of complexing agents as a function of the phase of growth—From his review of the literature and his own experiments, Sharp (1977) concluded that, generally, no significant accumulation of organic matter occurs during exponential growth but that significant accumulation does occur during stationary phase. In experiments with three chlorophytes (*P. morum, Chlamydomonas* sp., and *G. gigas*) and two cyanophytes

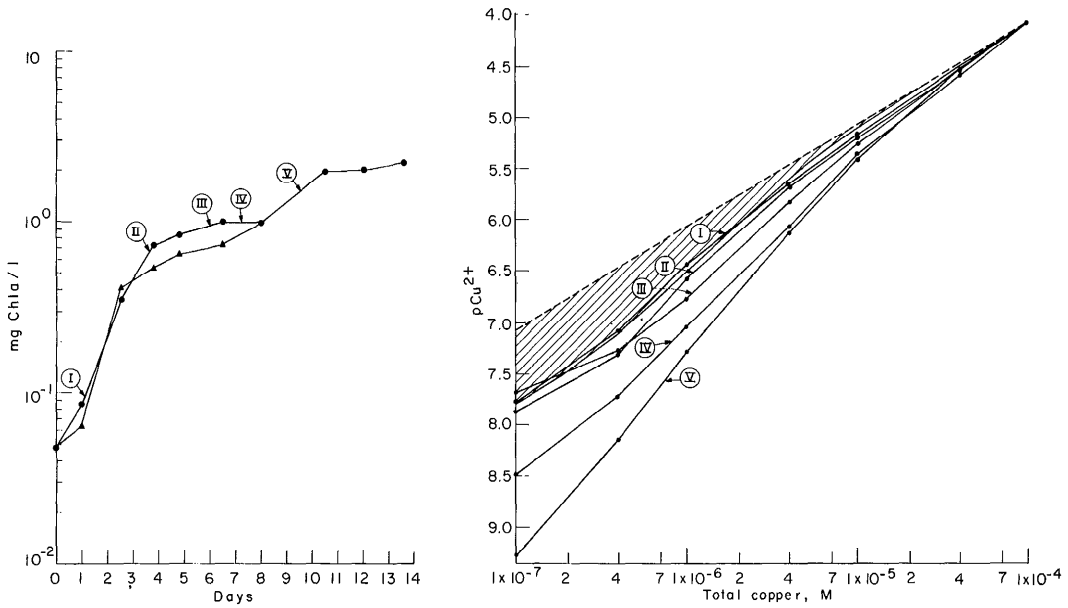

Fig. 7. As Fig. 6, but for *Anabaena flos-aquae*. Hatched area represents limits of detection of method for weak ligands.

(*A. flos-aquae* and *A. nidulans*) we found no measurable production of copper-complexing agents in the exponential growth phase but increasing concentrations of copper-complexing agents during slow growth or stationary phase (Figs. 6, 7, 8). These results support the hypothesis that the release of copper-complexing agents in batch cultures follows the same pattern as the general release of organic matter by algae. In terms of rate of release by individual cells, the results are somewhat inconclusive because the lack of sensitivity of the method does not provide enough data at low cell densities.

Figure 6 shows the increase in chlorophyll *a* with time in duplicate cultures of *Chlamydomonas* sp. and the corresponding copper titrations of samples removed at the times indicated. There was no measurable copper-complexing agent in a sample taken late in exponential growth (day 3, titration I). In a sample taken early in stationary phase (day 5, titration II), 1.8×10^{-6} M of weak complexing agent was measured, 4.5×10^{-6} M in a sample taken late in stationary phase (day 10, titration III). In poststationary phase (day 14, titration IV), cell lysis was observed and the concentration and strength of the copper-complexing agents in the culture medium had increased considerably.

In time-course experiments with *P. morum* and *G. gigas*, the release of copper-complexing agents appeared to be influenced by morphological changes associated with stationary phase, e.g. the concentration of complexing agent in *G. gigas* culture medium increased abruptly when mucilage-encased, coccoid cells became larger biflagellate cells. However, the results were similar to those above in that weak copper-complexing agents were excreted predominantly during stationary phase.

The time-course experiments with *A. flos-aquae* and *A. nidulans* are shown in Figs. 7 and 8. The removal of relatively large samples appears to have affected the growth of the cultures; nonetheless the rapid exponential growth was clearly followed by a period of slow exponential growth, as is typical for these algae. No

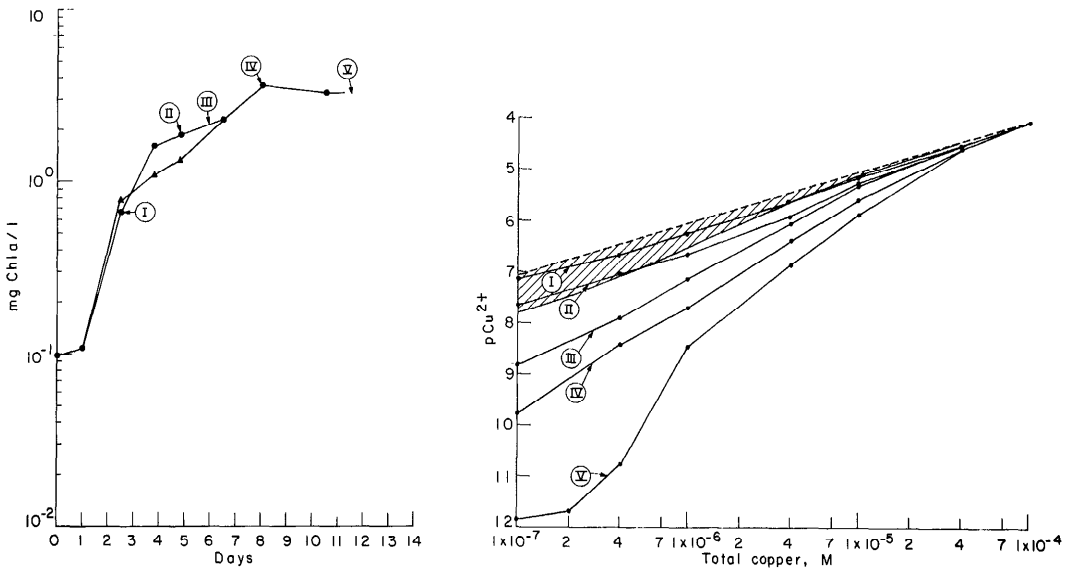

Fig. 8. As Fig. 6, but for *Anacystis nidulans*. Hatched area represents limits of detection of method for weak ligands.

complexing agent was detected during rapid growth and increasing concentrations of complexing agent are produced during slow growth by both cyanophytes.

The sequence of copper titrations in the time-course experiment with *A. nidulans* (Fig. 8) shows that very strong complexing agent is released mostly during stationary phase and suggests that two different ligands with different copper-complexing properties may be produced. For example, there is no increase in chlorophyll *a* concentration during the 3 days between titrations IV and V, and yet the cupric ion activity at 10^{-7} M total copper decreases from $10^{-9.8}$ M in titration IV to $10^{-11.5}$ M in titration V.

Discussion

By complexing metals and reducing the activities of metal ions, extracellular algal products could change the speciation of metals and possibly detoxify the cell's external milieu (Steemann Nielsen and Wium-Anderson 1971). The experimental results presented here characterize the copper-complexing capacity of organic compounds produced by 20 algal species in batch culture. From these results the ecological role of extracellular algal products as copper-detoxifying agents can be discussed quantitatively and several general conclusions can be drawn.

Weak copper-complexing agents are excreted by most of the eucaryotic algae studied. By improving the sensitivity of the methods of Swallow et al. (1978), we have found that their generally negative results do not extend to lower concentrations of complexing agents. The complexing agents can all be characterized by $\log\{{}^*K_{CuL}\} = 0.5$ and by 1×10^{-6} M $< L_T < 3 \times 10^{-6}$ M. The small variability among eucaryotic algae suggests that the complexing agent concentration may be controlled by the chemical conditions of the growth medium.

We expect the copper-complexing properties of extracellular metabolites from marine algae to be similar to those from freshwater algae. This is true at least for the four estuarine species that were studied in freshwater medium. Because of the interference of Cl^- ions with the cupric ion electrode (Westall 1977) a de-

salting step would have to be included before the methods presented here could be applied to marine algae.

The cyanophytes studied are different from the eucaryotic algae in that four of them produce strong copper-complexing agents ($^{c}K > 10^{7.5}$) during slow growth and stationary phase. Most likely two or more ligands with distinct concentrations and formation constants are present, and the pH dependence of copper complexation is not simple. Since for the three other blue-green algae studied we found only weak copper-complexing agents, we cannot make generalizations about the production of complexing agents by species from different classes of the cyanophyceae. There are apparent differences between species from the same genus: *A. flos-aquae* exudes strong copper-complexing agents, whereas *A. cylindrica* exudes weak complexing agents under the same culture conditions. *Anabaena cylindrica* was the species found by Fogg and Westlake (1955) to produce polypeptides that complexed copper. Whether the two results are in contradiction cannot be resolved because of differences in techniques and in strains of *A. cylindrica*.

The production of strong copper-complexing agents by blue-green algae is only one of many traits not shared with the other algae. Bound hydroxamates were not detected in any eucaryotic culture but were present in greater than micromolar concentrations in all cyanophyte cultures, suggesting that the ability to produce both strong iron- and copper-complexing agents is associated with the procaryotic nature of the cyanophyceae. Furthermore, since the formation constants for copper-hydroxamate siderochrome complexes are about 10^{8} (Anderegg et al. 1963), the possibility remains that the strong copper-complexing agents produced by some blue-greens are hydroxamate siderochromes.

As pointed out by Sharp (1977), it is important to consider possible artifacts in experiments on the excretion of organic matter by algae. Cell rupture during filtration would release compounds to the filtrate that would not be there if the sample had not been filtered. Although we feel that our results are not due to an artifact of filtration, we agree with Sharp that little can be done to completely avoid the problem and that no appropriate controls are obvious. Bacteria could also affect results by metabolizing extracellular algal products, releasing their own extracellular products, or attacking algal cells and making them "leakier." We found no bacterial contamination in the eucaryotic algal cultures. When samples from blue-green algal cultures were inoculated into Bactopeptone solutions and stored in the dark, the test tubes inoculated with coccoid species were clear after 4 days and those inoculated with filamentous species were clear for 3 days. Chrost (1978) found that extracellular metabolites released by *Chlorella vulgaris* in stationary phase were not assimilated by bacteria present in the algal culture, which also makes it doubtful that bacteria significantly affected our results.

In time-course experiments with three chlorophytes and two cyanophytes, copper-complexing agents were accumulated mainly during the stationary phase of batch cultures. Although no complexing agents were detected during exponential growth, the excretion of complexing agents more dilute than the detection limit of the method (about 2×10^{-7} M for strong ligands) could modify trace metal speciation significantly during exponential growth. In one culture (*A. nidulans*: Fig. 8), a significant release of complexing agent was measured without a concomitant increase in chlorophyll concentration. This is evidence that the release of strong complexing ligands observed principally in stationary phase is related to the physiological state of the cells, not just to the high cell density. More sensitive methods are needed to quantify the possible excretion of chelating agents during exponential growth. However the bulk of the complexing agents—weak and strong—appeared in batch culture media during stationary phase, not during lag phase or early exponential growth. This is inconsistent with the hy-

pothesis that they are released with the function of conditioning the medium.

The substantial excretion of copper-complexing agents by eucaryotic algae and blue-green algae in stationary phase of batch cultures raises the question of whether algal populations in physiological states corresponding to the stationary phase of batch cultures ever exist in nature. If batch cultures effectively mimic algal blooms, then the results presented here are relevant to the discussion of the chemistry of copper in natural waters. Copper-complexing agents are probably present during algal blooms in freshwater lakes, and there may be major differences in the nature of these complexing agents during diatom and chlorophyte blooms in the spring and cyanophyte blooms in the fall.

For a thorough evaluation of the role of copper-complexing algal exudates in natural waters, a number of questions must be answered. One is that of the relationship between cell concentrations in blooms of eucaryotic or blue-green algae and the concentrations of copper-complexing agents. Nutrient limitation and environmental conditions may be controlling factors. We can reason that since algal concentrations in natural waters are generally several orders of magnitude lower than in batch cultures, "typical" concentrations of algal-complexing agents in natural waters would also be several orders of magnitude lower than the micromolar concentrations found in batch cultures, i.e. between 10^{-7} and 10^{-9} M. However, in dense, surface blooms of filamentous blue-green algae in small ponds, concentrations of copper-complexing agents could be as high as 10^{-6} or 10^{-5} M. Such extreme conditions are of particular interest since treatment of blue-green algal blooms with copper sulfate is common practice.

Another question is how rapidly these organic compounds will be metabolized by bacteria or otherwise degraded in natural waters, and further experiments are necessary.

Whether the copper-complexing agents released by algae have ecological significance is determined by how their presence changes the speciation of copper in natural waters. One of the most critical parameters is the ratio of the concentration of algal complexing agent to the total concentration of copper. Natural concentrations of copper in freshwaters vary with depth and season in the same lake and from lake to lake in the range of 10^{-8} to 5×10^{-6} M, with an average value of 5×10^{-7} M reported in earlier studies (Riley 1939; Hutchinson 1957). In lakes and reservoirs treated with copper sulfate for algal control, initial copper concentrations in surface waters are about 10^{-5} M and are probably greater than concentrations of copper-complexing algal metabolites. Much of the filterable copper in lake water is known to be associated with dissolved organic compounds and not to be in solution as the free ion (Riley 1939; Hutchinson 1957; Gächter et al. 1973, 1978; Elder and Horne 1978).

We can best understand the effects of complexing agents by considering some simple examples in which the concentration of complexing agent exceeds the concentration of copper. The effect of 10^{-6} M of a weak organic acid ($\log\{{}^{*}K_{CuL}\} = 0.5$) on the speciation of 10^{-7} M copper in an idealized freshwater medium in equilibrium with the atmosphere was computed for typical pH values; the results are presented in a log concentration vs. pH diagram (Fig. 9). Over the pH range 6.0 to 8.5 the major copper species is the copper-eucaryotic algal metabolite complex; the decrease in $\log\{Cu^{2+}\}$ with pH is about 1:1, as is the increase in $\log\{CuCO_3(aq)\}$ with pH. The concentration of total copper chosen for the computation, 10^{-7} M, is close to the reported average concentration and is representative of freshwater lakes in general. However, the concentration of eucaryotic algal complexing agent, 10^{-6} M, should be representative of eutrophic lakes during or after dense chlorophyte or diatom blooms. The possible precipitation of tenorite and malachite was included in the computations but probably would not occur. The adsorption of copper by suspended particulates or colloids

Table 4. Effect of 10^{-6}M strong complexing agents excreted by two cyanophytes on speciation of 10^{-7}M copper at pH 6.2 in idealized freshwater in equilibrium with atmosphere.

	Anabaena flos-aquae	*Anacystis nidulans*
Conditional formation constant at pH 6.2	$^cK = 10^8$	$^cK = 10^{10}$
Chemical species, log{concn}		
Cu^{2+}	−9.0	−11.0
CuL	−7.0	−7.0
$CuCO_3$(aq)	−11.4	−13.4
$CuSO_4$(aq)	−10.5	−12.5
$CuCl^+$	−11.7	−13.7
$CuHPO_4$(aq)	−13.1	−15.1
$CuOH^+$	−10.7	−12.7
L^{2-}	−6.05	−6.05

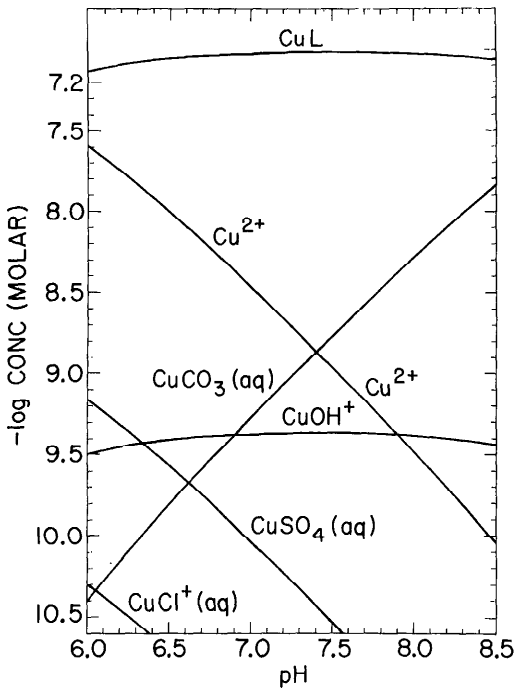

Fig. 9. Calculated −log concentration vs. pH diagram 10^{-6} M weak organic acid ($\log\{^*K_{CuL}\} = 0.5$) and 10^{-7} M copper in idealized freshwater ($Ca^{2+} = 2.5 \times 10^{-4}$ M, $Mg^{2+} = 1.5 \times 10^{-4}$ M, $Na^+ = 1.3 \times 10^{-3}$ M, $K^+ = 1 \times 10^{-4}$ M, $SO_4^{2-} = 1.5 \times 10^{-4}$ M, $H_3PO_4 = 1 \times 10^{-7}$ M, $Cl^- = 5 \times 10^{-4}$ M, $NO_3^- = 1 \times 10^{-3}$ M and $H_2SiO_3 = 1 \times 10^{-4}$ M) in equilibrium with the atmosphere.

and complexation by other organic compounds was not included because these processes are more difficult to quantify. Adsorption and complexation by humic compounds can affect the speciation of copper significantly in natural waters and could compete for copper with algal-complexing agents (Riley 1939; Hutchinson 1957; Manning and Ramamoorthy 1973; Vuceta 1976).

We made similar computations to examine the effect of 10^{-6} M strong copper-complexing agents from blue-green algae on the speciation of 10^{-7} M copper in idealized freshwater at pH 6.2. The omission of adsorption and complexation by other organic compounds from the computations is legitimate since these processes are not likely to compete for copper with the strong complexing agents. We expect the complexation of copper by cyanophycean exudates also to be pH-dependent, but the exact dependence was not determined experimentally. The formation constants calculated from the copper titrations are valid only near pH 6.25, and for this reason the computations are not presented for a broad pH range as in Fig. 9. The conditional formation constant of 10^8 is representatitve of the complexing agent produced by *A. flos-aquae* and the constant of 10^{10} is representative of the complexing agent produced by *A. nidulans*. For both values of ^cK the copper-algal exudate complex is the major copper species, and the cupric ion activity is much lower than for the weak organic acid at the same pH (Fig. 9). From the computations presented in Fig. 9 and Table 4, we conclude that complexation by extracellular algal products can dominate the speciation of copper in natural waters. Our computations also suggest that the reported association of filterable copper with organic compounds may often be attributable to complexation with autochthonous algal metabolites.

The sensitivity to copper of the three marine species studied by Sunda and Guillard (1976) and Anderson and Morel (1978) ranged from cupric ion activities of $10^{-8.5}$ to 10^{-11} M. Comparison of these results with the computations in Fig. 9 and Table 4 suggests that complexation

of copper by algal metabolites will lower the cupric ion activity to values toxic only to the more sensitive species. It does not follow, however, that algal products act as metal-detoxifying agents in natural waters. From the results of the time-course experiments, we know that micromolar concentrations of copper-complexing agents cannot be expected until the algal populations have reached high concentrations and are no longer actively growing. A detoxifying role can be envisioned only if the organic copper-complexing compounds produced during an algal bloom are not rapidly degraded and maintain low cupric ion activities that allow the growth of subsequent algal populations.

Conclusions

Three major conclusions can be drawn from the experimental work presented here.

Most cultures of eucaryotic algae in stationary phase contain micromolar concentrations of extracellular weak organic acids that complex copper ($\log\{{}^{*}K_{CuL}\} = 0.5$).

Blue-green algae produce weak organic acids similar to those from eucaryotic algae, and also strong copper-complexing agents (${}^{c}K \geq 10^{7.5}$), during the later growth phases of batch culture.

The copper-complexing agents produced by both eucaryotic and blue-green algae may dominate the speciation of soluble copper in freshwater lakes during algal blooms.

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