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Copper complexation by siderophores from filamentous blue-green algae[1]

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Abstract

From our experimental evidence that iron limitation greatly increases the extracellular concentration of strong copper-complexing agents in cultures of *Anabaena flos-aquae* and *Anabaena cylindrica*, and that the iron-algal exudate complex is much more stable than the copper complex, we conclude that strong copper-complexing agents released by filamentous blue-green algae are siderophores. Further experiments demonstrate that siderophore excretion is not a mechanism by which blue-green algae overcome the toxic effects of micromolar copper additions. From estimates of concentrations of iron, copper, and siderophores in iron-limited blue-green algal blooms, it is predicted that the copper-siderophore complex is the major copper species. The copper-siderophore complex is probably not toxic and its presence in freshwaters may be advantageous to cyanophyte populations.

It has been shown in laboratory and field experiments that under iron limitation blue-green algae excrete strong iron-complexing agents—hydroxamate siderophores (Simpson and Neilands 1976; Murphy et al. 1976). (The term siderophore, formerly siderochrome, emphasizes the role of these compounds in microbial iron transport). Murphy et al. (1976) suggested that eucaryotic algae cannot assimilate iron from the iron-hydroxamate siderophore complex and that blue-green algae monopolize low iron concentrations by excretion of hydroxamate siderophores. In previous work we found that four blue-green algal species excreted strong copper-complexing agents ($^cK > 10^8$) and that eucaryotic algae only released weak copper-complexing agents ($^cK < 10^{7.5}$: McKnight and Morel 1979). We also detected bound hydroxamate in the blue-green algal culture media and found the conditional formation constant for a copper-siderophore complex ($10^{8.4}$) to be similar to constants for strong copper-cyanophycean exudate complex (10^8–10^{12}). These results led to the hypothesis that the strong copper-complexing agents produced by cyanophytes are hydroxamate siderophores.

The work presented here, which tests this hypothesis, relies on what is already known about iron metabolism and siderophore production in microorganisms (Neilands 1973; Emery 1974). Besides cyanophytes, bacteria, fungi, and yeast have been found to produce either hydroxamate or phenolate siderophores (Neilands 1973). The role of siderophores in the uptake of iron by microorganisms was demonstrated in studies with iron mutants of *Salmonella typhimurium* (Luckey et al. 1972) and *Bacillus megaterium* (Davis and Byers 1971). Simpson and Neilands (1976) first identified a trihydroxamate siderophore, called schizokinen, in media from iron-starved cultures of *Anabaena* sp. Bound hydroxamate, indicative of hydroxamate siderophores, was detected in laboratory cultures of *Anabaena flos-aquae* and field samples from an *Anabaena* bloom by Murphy et al. (1976). Evidence of Fe (III)-complexing agents has been found both in coastal blue-green algal mats and in sea grass beds by a bioassay technique that uses a siderophore auxotroph, *Arthrobacter* JG-9 (Estep et al. 1975). Neilands (1973) provided a concise and useful outline of the mechanisms involved in microbial iron transport and

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pointed out that under conditions of low iron stress, overproduction of the ligand might cause a substantial number of the metal-free molecules to be displaced from the cell to the medium. The general stimulation of siderophore release by iron limitation provides a means to test our hypothesis that strong copper-complexing agents from blue-green algae are siderophores.

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Methods

Blue-green algal culture—Two filamentous blue-green algae, *Anabaena flos-aquae* (UTEX 1444) and *Anabaena cylindrica* (UTEX 1611), and one coccoid species, *Synechococcus leopoliensus* (UTEX 625, formerly *Anacystis nidulans*), were grown in 500 ml of WC medium (Guillard 1975) without synthetic chelators and with trace metals as in Aquil (Morel et al. 1979*b*). McKnight and Morel (1979) grew the same isolates of the filamentous species in WC medium; however a different isolate of *S. leopoliensus* was grown in Allen medium. *Anabaena flos-aquae* and *S. leopoliensus* produced strong copper-complexing agents and *A. cylindrica* produced weak copper-complexing agents. All cultures were grown at 20°C under continuous light, 20 μEinst$\cdot$cm$^{-2}\cdot$s^{-1}, in acid-washed 1-liter polycarbonate Erlenmeyer flasks. Sterile techniques were used throughout. Iron (10^{-5} M and 10^{-8} M) was added immediately before inoculation from freshly prepared solutions of $FeCl_3$. Copper was added during exponential growth from freshly prepared filter-sterilized solutions of $Cu(NO_3)_2$. In the initial experiments the medium was passed through a Chelex-100 column (Morel et al. 1979*a*) before addition of the trace metals and then autoclaved. The cultures were inoculated with 50-ml samples from late exponential phase cultures grown in Chelex-treated medium with 10^{-7} M iron. In the copper toxicity experiment non-Chelexed medium was sterilized by filtration and inoculated with 20 ml of an exponential phase culture with no added iron. Samples were taken from cultures of the same species at the same time, about 3 to 5 days after the end of exponential growth, and prepared for analysis as in Swallow et al. (1978).

Titrations—Copper titrations were performed as described by McKnight and Morel (1979). A combined copper-iron titration was developed to demonstrate the greater stability of the iron-siderophore complex. The copper titration is taken as far as 10^{-6} M total copper and then iron is added incrementally from freshly prepared 10^{-3} M and 10^{-2} M $FeCl_3$ solutions, going from 10^{-6} M to 3×10^{-5} M total iron. As the iron concentrations approached the equivalence point of the previous copper titration, the cupric ion activity, as measured by a cupric ion electrode, increased by several orders of magnitude. The disassociation of the copper complex on addition of iron was always fast, with equilibration in 10–15 min. The increasing cupric ion activity reflects the disassociation of the copper-siderophore complex on formation of the iron-siderophore complex. The formation of the iron-siderophore complex is also inferred from the development of the yellow color characteristic of iron trihydroxamates.

An iron interference for the cupric ion electrode has been reported by Hulanicki et al. (1977). In our own experiments an anomolous increase in electrode response of 75 mV was observed when 10^{-5} M iron was added to 10^{-6} M copper solutions at pH 4 in the absence of complexing agents. The iron interference decreased (to only 34 mV) when the stirrer was turned off, showing that the iron interference is probably caused by a reaction at the surface of the electrode. However, at the P_{CO_2} and pH of the titration, 10^{-2} atm and 6.25, the electrode response at 10^{-6} M copper increased 3 mV one minute after addition of 10^{-5} M iron and then gradually decreased 6 mV (presumably because of adsorption of copper on the fresh ferric hydroxide precipitate). We conclude that the interference effect of Fe^{3+} is small under the conditions of the titration. The method for demonstrat-

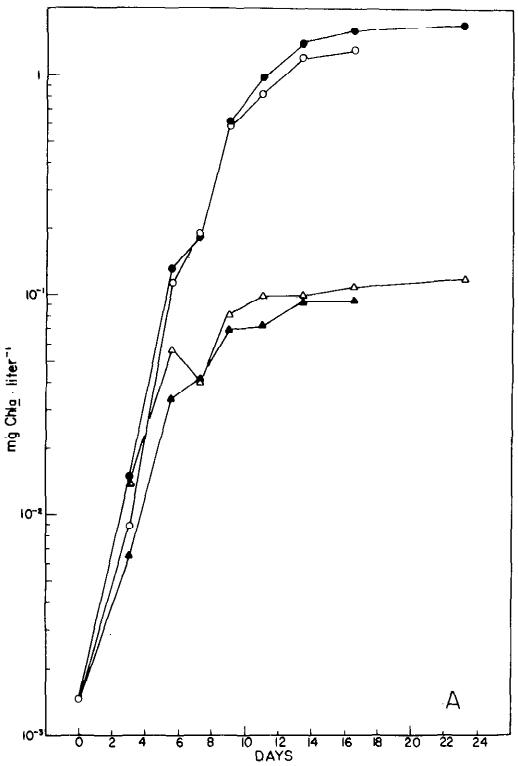
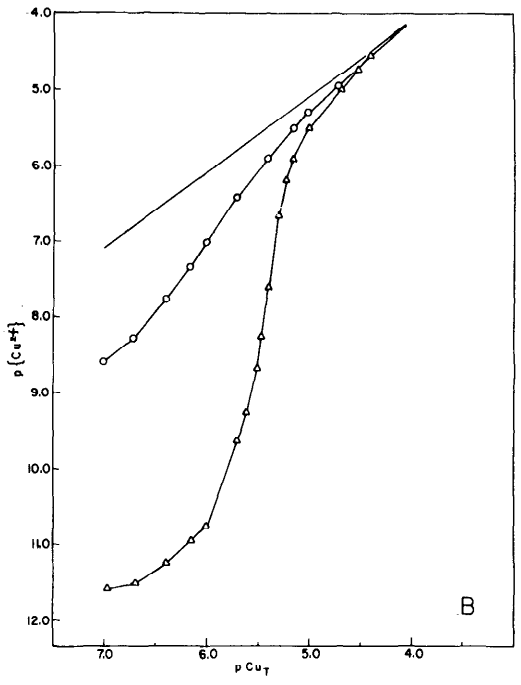


Fig. 1. A—Growth curves for duplicate cultures of *Anabaena cylindrica* with (○,●) 10^{-5} M and (△,▲) 10^{-8} M total iron. B—Copper titrations of *A. cylindrica* culture medium from cultures with (○) 10^{-5} M and (△) 10^{-8} M total iron.



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ing the stability of the iron-algal exudate complex was verified later with a 10^{-5} M solution of Desferal, (desferrioxamine B, a hydroxamate siderophore produced by CIBA-GEIGY). Because of precipitation effects beyond the equivalence point of the iron titration, the method cannot be used to detect weak complexing agents that bind copper more strongly than iron.

Analysis—Chlorophyll *a* was measured in a Turner fluorometer (Strickland and Parsons 1972). Dry weight was determined by the method of Sorokin (1973). Copper was measured by flameless atomic absorption spectrophotometry. The Csáky (1949) method as modified by McKnight and Morel (1979) was used to detect bound hydroxamate. So-

Table 1. Summary of chlorophyll *a*, biomass, and copper-complexing ligands in stationary-phase blue-green algal cultures with high and low iron concentrations.

	Total iron (M)	Chl *a* (mg·liter⁻¹)	Dry wt (mg·ml⁻¹)	Chl *a*/ Dry wt (mg·g⁻¹)	Total ligand concn (M)	°K†
Anabaena cylindrica	10^{-5}	1.7	0.117	14.5	3×10^{-6}	$10^{7.2}$
	10^{-8}	0.12	0.020	6.1	5×10^{-6}	$10^{10.2}$
Anabaena flos-aquae	10^{-5}	0.58	0.047	12.4	$\approx10^{-6}$*	—
	10^{-8}	0.06	0.018	3.4	1.5×10^{-5}	$10^{8.4}$
Synechococcus leopoliensus	10^{-5}	1.4	0.087	16.1	3×10^{-6}	$10^{7.0}$
	10^{-8}	0.09	0.017	5.2	undetectable	—

* Approximate value from McKnight and Morel (1979).
† °K is conditional formation constant for reaction $Cu^{2+} + L^- \rightleftharpoons CuL^+$ at pH 6.25.

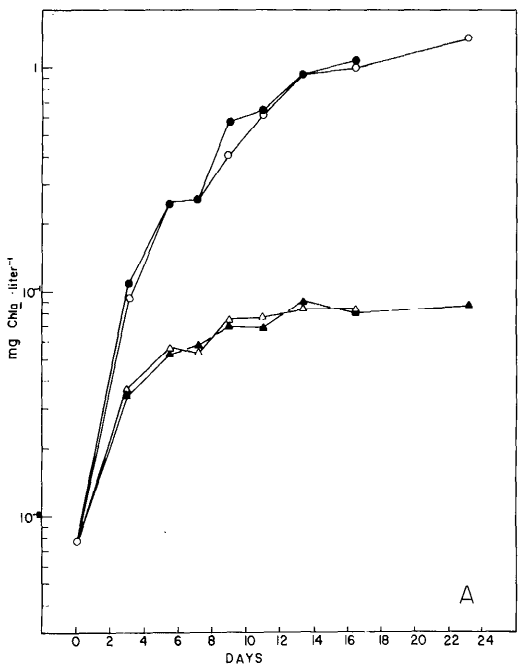
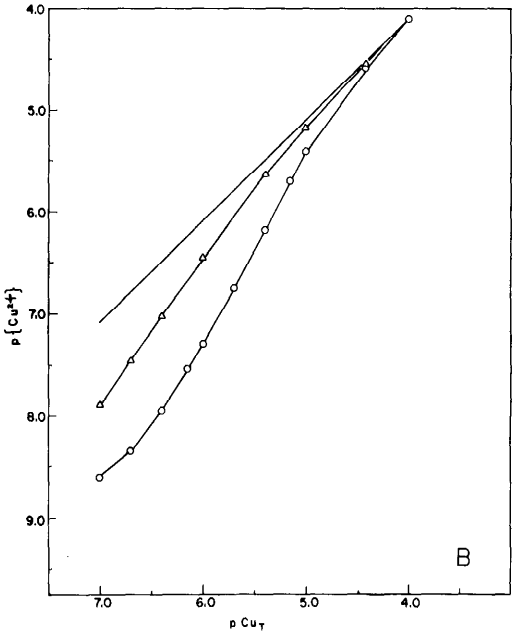


Fig. 2. As Fig. 1, but for *Synechococcus leopoliensus*.



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lutions of Desferal (10^{-7}–10^{-5} M) were used as standards for the Csáky test. At Desferal concentrations $>10^{-6}$ M, the hydrolysis step (6 h at 100°C in 3 N acid) was not complete. Since other hydroxamate siderophores may react differently, the Csáky method is not used as a precise quantitative test for bound hydroxamate.

Results

The siderophore hypothesis was tested by seeing whether the production of strong copper-complexing agents by blue-green algae was enhanced by iron limitation. The copper titration of media from high (10^{-5} M) iron and low (10^{-8} M) iron cultures of *A. cylindrica* and *S. leopoliensus* and the corresponding growth curves are shown in Figs. 1 and 2. Table 1 summarizes for all three algae the concentration of chlorophyll *a* and dry weight, and their ratio, during stationary phase and the equivalent ligand concentrations and conditional formation constants for the copper-algal exudate complexes. Only the low iron *Anabaena* cultures had greater than micromolar concentrations of strong copper-complexing agents (${}^{c}K>10^{8}$). On the other hand, all the cyanophyte cultures gave positive results to the Csáky test, which is indicative of hydroxamate siderophores. The fact that only iron-free siderophores will complex copper explains in part the poor correlation between the detection of bound hydroxamates and strong copper-complexing agents.

All the low iron cyanophyte cultures were chlorotic and had lower dry weights. Iron deficiency causes chlorosis in algae and higher plants (Price and Carell 1964; Price 1968). Oquist (1971, 1973) showed that iron deficiency in *S. leopoliensus* induces a spectral shift in the chlorophyll *a* absorption peak and a decrease in the photosynthetic capacity of photosystem I. Morphological changes were also observed in iron-limited *Anabaena* cultures (McKnight 1979); in *A. flos-aquae* cultures there were many (2×10^{6} cells·ml^{-1}) akinetes, and in *A. cylindrica* cultures the chains were short and clumped together.

The results of the copper titrations for

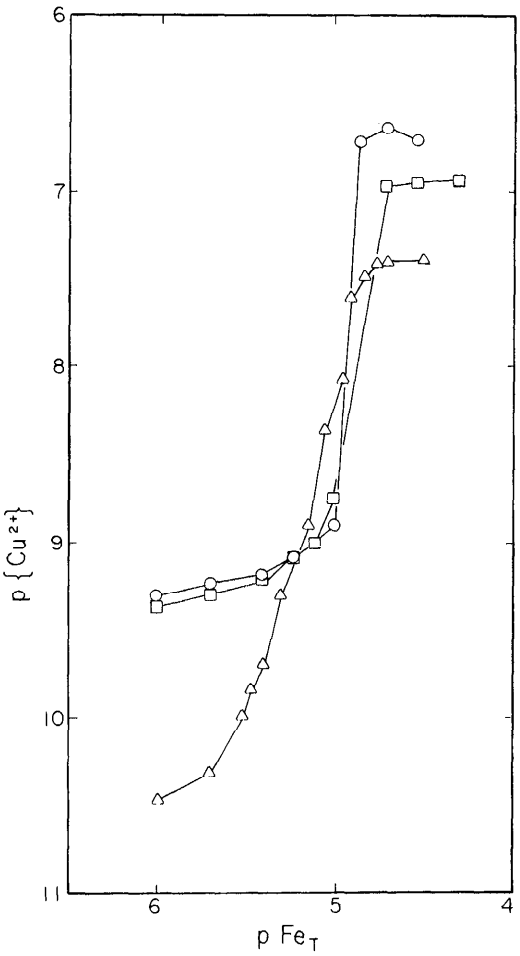

Fig. 3. Combined copper and iron titration of medium from low iron cultures of *Anabaena flos-aquae* (○) and *Anabaena cylindrica* (△) and of 10^{-5} M solution of Desferal (□).

A. cylindrica clearly demonstrate the release of some strong copper-complexing agent, presumably a siderophore, in response to iron limitation. As in our previous work, only weak copper-complexing agents ($^cK<10^{7.5}$) were detected in iron-rich cultures. But in iron-poor cultures, 5×10^{-6} M of a complexing agent with a conditional formation constant of $10^{10.3}$ was released. The results for the other filamentous blue-green alga, *A. flos-aquae*, were inconclusive because in several copper titrations of medium from the iron-rich culture, acceptable chemical equilibration failed to occur 24 h after addition of 10^{-7} M copper. We have previously found copper titrations of media from cultures of *A. flos-aquae* with 10^{-5} M iron added to be highly reproducible. The different result in this experiment is probably associated with the iron-depleted inoculum. The release of 10^{-5} M strong copper-complexing agent in the iron-limited culture is more than an order of magnitude greater than the release in previous iron-rich cultures and is certainly consistent with the siderophore hypothesis.

For the coccoid cyanophyte, *S. leopoliensus*, the copper titrations (Fig. 2) show that the excretion of weak copper-complexing agents is not stimulated, but depressed by iron limitation. This indicates that the weak complexing agents excreted by *S. leopoliensus* under these culture conditions are not siderophores. The lower concentration in the iron-limited culture is probably due to the lower biomass. The results for *S. leopoliensus* imply that weak copper-complexing agents ($^cK \leq 10^{7.5}$) produced by other cyanophytes and eucaryotic algae are not siderophores. The possibility remains, however, that the strong copper-complexing agents ($^cK \geq 10^{10}$) previously detected in cultures of coccoid cyanophytes grown in Allen medium (McKnight and Morel 1979) were hydroxamate siderophores.

The siderophore hypothesis is further supported by the results of the combined copper and iron titrations of media from low iron cultures of *A. cylindrica* and *A. flos-aquae* (Fig. 3). In the *Anabaena* culture media and in 10^{-5} M of Desferal, the cupric ion activity increases several orders of magnitude as the added iron displaces copper from the siderophore. These results show that the formation constants for the iron-algal exudate complexes are at least an order of magnitude greater than those for the copper complexes. The conditional formation constants for the copper-Desferal complex and the copper-*A. flos-aquae* exudate complex are indistinguishable ($^cK \cong 10^{8.3}$–$10^{8.4}$) and the combined titrations of low iron *A. flos-aquae* culture medium and 10^{-5} M Des-

feral are also very similar. On the other hand, the conditional formation constant for the copper-*A. cylindrica* complex is much higher (${}^{c}K \cong 10^{10.2}$) and presumably reflects a different chemical structure.

Fogg and Westlake (1955) showed that copper toxicity to *A. cylindrica* was repressed by addition of extracellular polypeptides from previous stationary-phase cultures. It cannot be determined whether the extracellular polypeptides in their study would correspond to weak or strong copper-complexing agents. However, their work suggests that siderophores may play a role in the response of cyanophytes to copper toxicity. The design of an experiment to study the relationships between excretion of siderophores and copper toxicity is complicated by chemical unknowns. We have advocated chemically well defined culture media where the free metal activities can be calculated with some certainty (Morel et al. 1979*b*). The methods rely on micromolar or greater concentrations of synthetic chelators to buffer metal activities, which are not reconcilable with potentiometric detection of algal-excreted copper-complexing agents. On the other hand, in the absence of chelators, adsorption of copper on ferric hydroxide may control the speciation of copper in an unpredictable way and confound the interpretation of copper toxicity experiments in chelator-free media. Furthermore, in low iron medium, copper speciation will probably be controlled not by adsorption but by the gradual accumulation of extracellular siderophores.

Although limited by the ill defined chemistry of the culture medium, the results of a simple copper toxicity experiment do undermine the hypothesis of enhanced excretion of siderophores by cyanophytes in response to copper toxicity. In replicate low iron (10^{-8} M) cultures of *A. flos-aquae* which were spiked during exponential growth with 10^{-5} M and 10^{-6} M copper, there was rapid decrease in chlorophyll *a* and no recovery (Fig. 4). Most of the copper (60%) remained in solution at both added copper concentrations and was weakly bound,

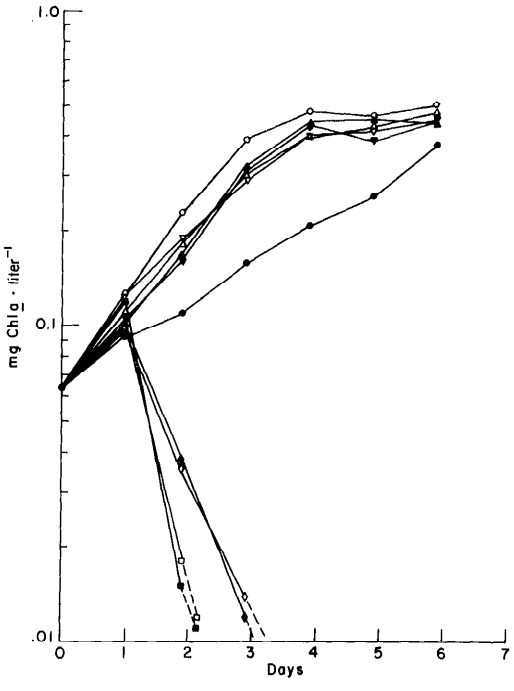

Fig. 4. Response of replicate cultures of *Anabaena flos-aquae* to copper addition during exponential growth phase: ■,□—10^{-5} M; ◆, ◇—10^{-6} M; ●,○—10^{-7} M; ▲,△—10^{-8} M; ▼,▽—10^{-9} M total copper.

probably by cellular debris (Table 2). The test for bound hydroxamate was also negative for the copper-stressed cultures, further demonstrating that high concentrations of detoxifying siderophores are not released by *A. flos-aquae* when stressed with copper. The cultures receiving copper spikes of 10^{-7} and 10^{-8} M survived (Fig. 4) and produced about 10^{-5} M of a strong copper-complexing agent and bound hydroxamate (Table 2) as did the control cultures and the other low iron culture of *A. flos-aquae* (Table 1). The lower chlorophyll *a* concentration in previous cultures probably reflects lower iron contamination in the Chelex-treated medium. The excretion of 10^{-5} M siderophore in the surviving *A. flos-aquae* cultures shows that eventual excretion of sufficient copper-complexing agent to bind the 10^{-5} and 10^{-6} M copper spikes was prevented by the immediate death of the cultures and not by

Table 2. Results of *Anabaena flos-aquae* copper toxicity experiment.

ΣCu added (M)	Particulate Cu* (M)	ΣCu in filtrate (M)	$\{Cu^{2+}\}$ in filtrate (M)	Bound hydroxamate	Equiv ligand concn (M) with $^cK = 10^{6.3}$
10^{-5}	1.7×10^{-6}	8.3×10^{-6}	7.9×10^{-7}		
10^{-6}	2×10^{-7}	7.3×10^{-7}	1.6×10^{-8}		
10^{-7}	—	4×10^{-7}	—	+++†	1.3×10^{-5}
10^{-8}	—	—		+++	1.5×10^{-5}
None	—	—		+++	1.5×10^{-5}

* Retained on 0.4-μm filter sample filtered 2 days after copper addition.
† +++ is ca. 1×10^{-5} M Desferal equivalents.

other factors such as the health of the inoculum or nutrient limitation.

Discussion

In previous work we concluded from simple chemical equilibrium computations that strong copper-complexing agents from blue-green algae may control the speciation of copper in lakes during or after blooms (McKnight and Morel 1979). Our new results do not change that basic conclusion. In fact, our original estimate of 10^{-6} M ligand being released in algal blooms appears reasonable now that we have found 5×10^{-6} and 10^{-5} M ligand in cultures with chlorophyll *a* concentrations close to those in field samples from algal blooms.

Iron is an important micronutrient in chlorophyll synthesis and in N_2 fixation (Stewart 1973) and its availability has been shown to control phytoplankton succession from green to blue-green algae in bioassay experiments (Lange 1971; Morton and Lee 1974; Murphy et al. 1976; Elder and Horne 1977). Lange (1971) followed the collapse of an *Aphanizomenon* bloom in Lake Erie when nitrogen and phosphorus concentrations were increasing but dissolved iron concentrations had decreased to $<2\times10^{-8}$ M. In limnocorral experiments, high rates of nitrogen fixation in an *Anabaena* bloom were associated with high iron concentrations and rapid assimilation of ^{55}Fe; production of hydroxamate siderophores in the bloom was also demonstrated (Murphy and Lean 1975; Murphy et al. 1976). In a survey of 49 American lakes, the concentration of dissolved iron (passing through a 0.45-μm filter) ranged from 5×10^{-6} M to 1×10^{-7} M (Miller et al. 1974) and was generally less than the micromolar concentrations found to enhance blue-green algal dominance of phytoplankton assemblages in laboratory experiments. Taken as a whole such experimental results show that iron limitation of N_2-fixing blue-green algal blooms may be an important phenomenon and, by extension, that significant concentrations of iron-free siderophores may be present in freshwater lakes.

Whether the copper-siderophore complex is an important copper species in lakes primarily depends on the relative concentrations of iron, copper, and siderophores. Since siderophore complexes of cadmium, lead, nickel, cobalt, and aluminum also have formation constants $>10^8$ (Anderegg et al. 1963), they may also be important and should be considered in detailed calculations. Based on the literature and our own results, we speculate that dissolved iron concentrations near 10^{-7} M will limit populations of N_2-fixing blue-green algae and that these iron-limited populations will release concentrations of siderophores in the range 10^{-7}–10^{-5} M—greater than the available iron concentration. Copper concentrations of 10^{-7} M or greater can be taken as typical of freshwaters (Hutchinson 1953), although there may be a correlation between low concentrations of copper and low concentrations of iron. From these estimates we propose that relative concentrations of siderophores, copper, and iron in iron-limited blue-green algal blooms probably are: $(\text{siderophore})_T > (Cu)_T > (Fe)_T$. Although much of the copper would otherwise be com-

plexed by humic compounds or adsorbed on particulate material (Gächter et al. 1978; Vuceta 1976), at micromolar iron-free siderophore concentrations the copper-siderophore complex will be the dominant copper species.

The results of our copper toxicity experiments are not much help in predicting how the presence of significant concentrations of a copper-siderophore complex might affect phytoplankton ecology in eutrophic lakes. However, that is an important question since copper sulfate is widely used as an algicide to prevent nuisance blooms in lakes and reservoirs. Blue-green algae, especially filamentous bloom-forming species, are generally more sensitive to copper toxicity than chrysophytes, chlorophytes, and diatoms (Whitton 1973; Gibson 1972; Horne and Goldman 1974; Steemann Nielson and Brunn Larsen 1976).

The assumption that copper toxicity is a unique function of the cupric ion activity, as has been shown for marine eucaryotic algae (Sunda and Guillard 1976; Jackson and Morgan 1978; Anderson and Morel 1978), probably holds for iron-limited blue-green algae exposed to significant concentrations of copper-siderophore complex. The conformation of the hydroxamate siderophore in the vicinity of the metal ion is critical for recognition by the microbial iron-transport system. The different coordination numbers for copper(4) and iron(6) will result in different geometries for the copper-siderophore chelate (square planar) and the iron-siderophore chelate (octahedral: Emery 1971). By this argument, it appears unlikely that the copper-siderophore complex will be assimilated by blue-green algal cells. Emery (1971) also found that the copper-desferriferrichrome complex was not assimilated by the smut fungus *Ustilago sphaerogena* and that copper did not affect ferrichrome uptake. Murphy and Lean (1975) found that the biological uptake of ^{55}Fe by iron-limited *Anabaena* populations was blocked by addition of 10 μM copper; however, their work does not demonstrate toxicity of the copper-siderophore complex because the added copper was probably in excess of the extracellular siderophore concentration. Unless extracellular copper siderophore complexes interfere with iron assimilation indirectly, the presence of copper-siderophore complexes in freshwaters should be advantageous to cyanophyte populations in competition with eucaryotic algal populations because of the resulting decrease in cupric ion activity.

In summary, we have shown for two filamentous blue-green algae, *A. flos-aquae* and *A. cylindrica*, that extracellular strong copper-complexing agents are hydroxamate siderophores by demonstrating that increased production of strong copper-complexing agent is induced by iron limitation and that the iron-algal exudate complex is more stable than the copper complex. Final verification would be to demonstrate that the formation constant for the iron-algal exudate is about 10^{30} and that the algal exudate is capable of transporting iron in *Anabaena* mutants lacking a high-affinity iron transport system. The copper-siderophore complex is predicted to be the major copper species in blooms of N_2-fixing blue-green algae where iron is the limiting micronutrient. The copper-siderophore complex is not expected to be toxic to cyanophytes, and siderophores do not appear to be released in response to copper toxicity. Better experiments exploring the relationship between iron assimilation and copper toxicity in phytoplankton may explain why blue-green algae are ostensibly more sensitive to copper toxicity than are green algae and diatoms.

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