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Production of strong, extracellular Cu chelators by marine cyanobacteria in response to Cu stress

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Abstract

Copper speciation in the upper marine water column is dominated by strong ligands thought to be of recent biological origin. Cultures of the marine cyanobacteria *Synechococcus* spp., a ubiquitous and important group of phytoplankton highly sensitive to Cu toxicity, were previously shown to produce chelators comparable in strength to those detected in the water column. Here we show that cultures of *Synechococcus* exposed to toxic concentrations of Cu produce an extracellular ligand with a binding constant comparable to constants for ligands found in the water column. Coordination of Cu by this compound decreases the concentration of free cupric ion (the toxic form) in the culture media to levels that do not inhibit growth. A tight linear correlation between chelator and Cu concentration suggests that production of this substance may be regulated by the concentration of free Cu in the media in a feedback mechanism. Similarly, the concentrations of Cu and Cu-binding ligands in the water column are often closely related. These results suggest that cyanobacteria modify Cu chemistry in seawater, creating conditions more favorable for growth.

Considerable evidence suggests that the distribution and speciation of trace metals in the upper water column plays an important role in the species composition and physiology of phytoplankton assemblages (Sunda 1994 and refs. therein). Speciation is important because only certain forms of a given metal are biologically available. In the upper water column, speciation of many biologically active trace metals is controlled by complexation with strong organic ligands (Bruland et al. 1991; Sunda 1994). Complexation generally lowers the biological availability of a given metal because the free metal ions are the most biologically available forms (Sunda 1994). In addition, complexation may play an important role in the geochemical cycling of these elements in the upper ocean. For many elements, concentrations of these ligands are highest in the euphotic zone and decline to nondetectable values in deep waters (Bruland et al. 1991; Rue and Bruland 1995). This distribution suggests that the compounds are of recent biological origin and are not refractory compounds. However, their biological function, if any, is unknown.

Complexation is of particular importance for Cu, an essential micronutrient that is also toxic. Cu is complexed by low concentrations of a strong ligand having a concentration of 1–2 nM and a conditional stability constant of at least 10^{13} (van den Berg et al. 1987; Coale and Bruland 1990; Sunda and Huntsman 1991; Moffett 1995). However, the structure of the ligand or ligands is unknown, so it is generally referred to as ligand class 1 (L1).

Previously, we reported that cultures of the marine cyanobacterium *Synechococcus* produce a chelator with a similar conditional stability constant to water column L1 ligands (Moffett et al. 1990). *Synechococcus* spp. is an important constituent of oceanic phytoplankton assemblages (Iturriaga and Marra 1988; Olson et al. 1990) and is therefore a plausible source of strong Cu chelators in seawater. Moreover, *Synechococcus* is extremely sensitive to Cu toxicity compared to eucaryotic phytoplankton (Brand et al. 1986). In seawater, Cu would significantly inhibit *Synechococcus* growth rates even at nanomolar concentrations, were it not for the presence of L1. Because *Synechococcus* clearly benefits from the presence of L1 in the water column, production of a similar ligand in culture raises the possibility that *Synechococcus* may produce L1 to modify its environment to create conditions more favorable for growth. To test this hypothesis, we exposed cultures of *Synechococcus* to progressively higher concentrations of Cu to see whether this would stimulate production of strong chelators. We also did this with cultures of eucaryotic phytoplankton, even though eucaryotes did not produce strong chelators in the earlier study (Moffett et al. 1990). Our reasoning was that because these organisms are less sensitive to Cu, higher levels might be required to stimulate chelator production.

We found no information about the structure or coordination sites of L1 compounds, so we had no basis for designing a direct assay. Instead, the distribution of L1 compounds has been determined in the oceans by titrat-

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Table 1. *Synechococcus* clones used in this study.

Clone	Isolation location	Date
DC-2 (WH7803)	Sargasso Sea (33°44.9′N; 67°29.8′W)	1 Jul 78
A2357	Tongue of the Ocean, Bahamas	Nov 84
A2346	Bimini, Bahamas	18 Nov 84
A3844	Waquoit Bay, Massachusetts	12 Sep 93

Table 2. Major nutrients and micronutrients composition of culture media.

Nutrient	M concn	Nutrient	M concn
Silica	10^{-5}	Zn	10^{-8}
Nitrate	10^{-4}	Co	10^{-9}
Ammonia	10^{-5}	Se	10^{-9}
Phosphate	10^{-5}	Thiamin	10^{-7}
Fe citrate	10^{-6}	Biotin	10^{-8}
Mn	10^{-7}	B_{12}	10^{-8}
Na citrate	10^{-5}		

ing seawater samples with Cu and quantifying the extent of complexation by means of various speciation techniques. Our approach was to use the same techniques on filtrates from the cultures as had been used in the water column to allow us to directly compare culture and water-column data. Cu speciation measurements were performed on culture filtrate samples that had been titrated with Cu and analyzed using competitive ligand exchange cathodic stripping voltametry (CLE/CSV). With this approach, a synthetic ligand was added to the sample to compete with naturally occurring chelators for a fraction of the Cu. Provided that the conditional stability constant of the synthetic complex is known and its concentration can be measured, titration data can be used to derive estimates of the concentrations and conditional stability constants of naturally occurring ligands. CLE/CSV has become the most widely used ligand exchange method. Addition of the synthetic ligand leads to formation of a surface-active complex that can be detected by adsorptive cathodic stripping voltametry. The method has been used for several elements, including Cu (van den Berg 1984; van den Berg et al. 1987; Donat and van den Berg 1992). For this study, we used as competing ligands the β diketonate, benzoylacetone (bzac) (Moffett 1995), and salicylaldoxime (SA) (Campos and van den Berg 1994). A closely related β diketonate, acetylacetone (acac), was used in our previous study of ligand production by *Synechococcus* (Moffett et al. 1990) in a ligand exchange-solvent extraction technique.

Experiment

Axenic cultures of *Synechococcus* were prepared from four clones (Table 1) isolated from diverse coastal and oceanic locations and currently maintained in Larry Brand's culture collection at the University of Miami. Protocols for culturing the organisms are described by Brand et al. (1986). The growth medium was prepared from oligotrophic seawater (collected from NE Providence Channel, Bahamas). Table 2 shows the major nutrients and micronutrients added to this water. Other workers often add EDTA to growth media to chelate toxic metals. However, no EDTA was added to the growth media in these experiments because it would have interfered with detection of chelators produced by the algae. We found that by using oligotrophic seawater and acid-washed plastic containers, metal contamination could be minimized so that EDTA was not required for growth. In some experiments, 5 μM of NTA—a much weaker chelator—was used to provide a low background of Cu complexation. Cultures in the latter stages of exponential growth were spiked with 10–20 nM Cu every other day until we had a suite of samples with dissolved Cu concentrations in the media of 10–200 nM. These were filtered at 24–48 h after the last Cu addition and Cu titrations were performed on the filtrate to determine the concentration and conditional stability constants of Cu-complexing ligands. Cell densities of each culture were determined before filtration by epifluorescence microscopy. For *Synechococcus,* cell densities ranged from 1.5 to 2.0×10^7 cells ml^{-1}.

CLE/CSV measurements were made with a PAR 264A polarographic analyzer with a PAR 303A static mercury drop electrode. Cu titrations were performed as follows. Each culture filtrate was divided into 20-ml aliquots in 125-ml Teflon bottles. These were spiked with different concentrations of Cu (0–200 nM) and benzoylacetone (1–5×10^{-4} M). The solutions were generally allowed to equilibrate for 6–12 h before analysis, by which time steady-state values were obtained. For analysis, 10 ml of solution was transferred to a quartz sample cup and installed on the electrode, which was set to hanging drop mode. Instrument settings were: deposition potential, -0.08 V (vs. Ag/AgCl electrode); deposition time, $t_d = 1$ min; scan range, -0.08 to -0.5 V; scan rate, 10 mVs; drop time, 0.2 s; pulse height, 25 mV. Reduction of the copper benzoylacetone complex produces a well-defined peak at -0.27 V. Benzoylacetone was evaluated for this application by criteria established by others (van den Berg 1984; Donat and van den Berg 1992) for other CSV ligands (Moffett 1995). Several titrations also were performed with salicyladoxime as the competing ligand. Instrument settings and protocols with salicylaldoxime were identical to those described by Campos and van den Berg (1994).

Benzoylacetone (Aldrich) required purification before use. This was accomplished by recrystallization in aqueous EDTA solution (10^{-3} M) followed by double recrystallization in distilled water to remove EDTA. A solution containing 5×10^{-2} M benzoylacetone (hereafter referred to as bzac) in methanol was used as a stock solution. Salicylaldoxime (Aldrich) was purified by recrystallization in the same manner.

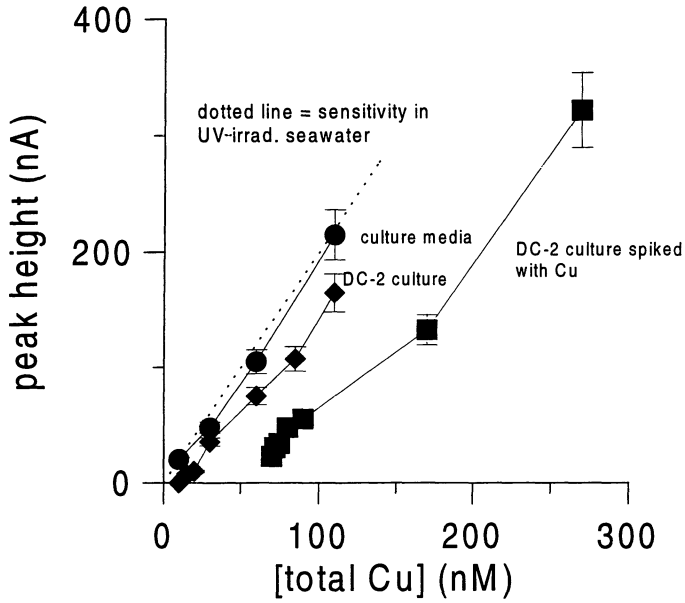

Fig. 1. Peak height at −0.27 V vs. Ag/AgCl electrode as a function of total dissolved Cu in solution for Cu titrations of culture media (initial Cu = 4 nM Cu), a filtered DC-2 culture (initial Cu = 10 nM), and a filtered DC-2 culture spiked with Cu (initial Cu = 70 nM). [bzac] = 5×10^{-4} M.

The side-reaction (α) coefficient for Cu benzoylacetone complexes, defined as ([Cu(bzac)] + [Cu(bzac)$_2$])/[Cu$^{2+}{}_f$], was determined at each benzoylacetone concentration used in the study (Moffett 1995). Side-reaction coefficients for Cu(bzac) are proportional to [bzac]^2 over the concentration range 1–5 × 10^{-4} M bzac, indicating that the Cu(bzac)$_2$ complex is the dominant electroactive species. For Cu(bzac)$_2$, log β_2 = 10.7, which was constant over the pH range 8.0–8.3—the range of values encountered in this study. Side-reaction coefficients for Cu complexes with SA were taken from Campos and van den Berg (1994). The side-reaction coefficient for Cu complexes with SA at 2×10^{-6} M SA (7.1×10^3) is similar to the side-reaction coefficient Cu(bzac)$_2$ at 5×10^{-4} M bzac (1.25×10^4), so some titrations were performed on the same samples at these ligand concentrations for comparative purposes.

The total dissolved Cu concentration in each sample was determined after UV oxidation of a 100-ml aliquot of seawater acidified to pH 2 with Ultrex HCl. The sample pH was adjusted to 7.7 with distilled ammonia and HEPES buffer and Cu analyzed by CSV with 2×10^{-4} M bzac.

Results

To determine ligand concentration and stability constant data from Cu titrations, one must know the fraction of Cu present as the Cu(bzac)$_2$ complex at each point on the titration curve. Therefore, the system must be calibrated accurately so that [Cu(bzac)$_2$] can be calculated from the peak current signal generated by the cathodic scan.

The peak current, i_p, is related to the concentration of Cu(bzac)$_2$ in solution by the equation

$$i_p = S[\mathrm{Cu(bzac)_2}] \quad (1)$$

where S is the sensitivity. S is readily determined in UV-oxidized samples by standard additions of Cu. However, in natural samples, S must be determined from the linear portion of the titration curve when all complexing ligands are saturated to distinguish the effects of ligand competition, which does not affect S, from surfactant interferences, which do (van den Berg 1984). Figure 1 shows i_p vs. Cu$_T$ for the growth media and filtrates of two *Synechococcus* cultures. In each sample, the data points fall well below the UV-oxidized seawater line, reflecting competition for much of the Cu by natural ligands that outcompete bzac. However, as the natural ligands become saturated, the slope approaches the slope in UV-oxidized water because virtually all of the Cu added subsequently is associated with bzac. Because the linear region of the titration plot is parallel to the UV-oxidized seawater line, S is the same in all these samples. This trend is more difficult to demonstrate for the Cu-spiked culture because above ~200 nA, S decreases due to a well-documented effect—the saturation of the Hg surface by ligand complexes (van den Berg 1984). Therefore, the slope approaches but is always smaller than the UV-oxidized slope. However, by running the samples at a shorter deposition time (where the peak current is proportionately lower) we can confirm that S is the same for all three samples.

The data shown in Fig. 1 indicate that the media, the *Synechococcus* culture, and the Cu-spiked culture all have detectable levels of Cu-complexing substances, but there is clearly much more of these substances in the Cu-stressed culture. The horizontal distance between these samples and the UV-irradiated seawater line provides an estimate of a lower limit of ligand concentration. However, a more detailed treatment of the data is required to determine ligand concentrations and conditional stability constants.

Data such as those in Fig. 1 yield the fraction of Cu complexed by bzac (and hence becoming electroactive) at different Cu concentrations. This process is related to the conditional stability constants and ligand concentrations of all ligands in the sample by the relationship

$$\frac{[\mathrm{Cu(bzac)_2}]}{[\mathrm{Cu_T}]} = \frac{\beta_2[\mathrm{bzac}]^2}{1 + \Sigma K_i \mathrm{L}_i + \beta_2[\mathrm{bzac}]^2}. \quad (2)$$

K_i is the conditional stability constant, L$_i$ the concentration of the ith natural ligand, ΣK_iL$_i$ the side-reaction coefficient for the naturally occurring ligands, and β_2[bzac]^2 the side-reaction coefficient for bzac complexes, which was determined against the model ligands. The side-reaction coefficient for all naturally occurring ligands (including inorganic ligands) is related to free cupric ion concentration by the relationship

$$\frac{[\mathrm{Cu^{2+}}_f]}{[\mathrm{Cu_T}] - [\mathrm{Cu(bzac)_2}]} = \frac{1}{1 + \Sigma K_i \mathrm{L}_i}. \quad (3)$$

Data in this study were analyzed with a single ligand model that was a nonlinear fit to a Langmuir adsorption

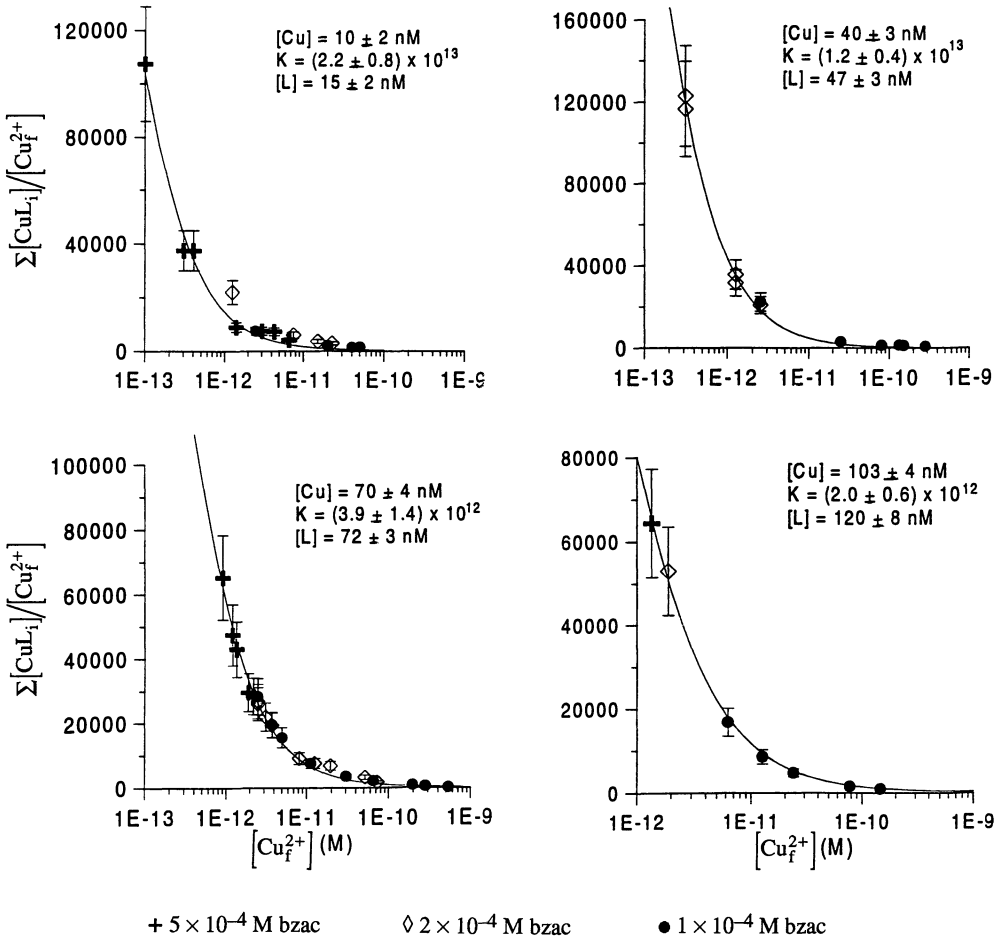

Fig. 2. Titration data plotted as reciprocal Langmuir isotherms for filtered DC-2 cultures containing different concentrations of Cu. Solid lines—best fit to the data with Eq. 5; note different scales.

isotherm. This model has been described previously by Gerringa et al. (1995). Those workers made a convincing case from a statistical perspective for selecting a nonlinear fit over linearization plots, such as van den Berg/Ruzic or Scatchard plots. The single ligand model is derived from

$$K = \frac{[\mathrm{CuL}]}{[\mathrm{Cu}^{2+}{}_f]\,[\mathrm{L}_f]} \tag{4}$$

where

$$[\mathrm{L}] = [\mathrm{L}_f] + [\mathrm{CuL}]. \tag{5}$$

Rearranging Eq. 4 and 5 yields a reciprocal Langmuir isotherm:

$$\frac{[\mathrm{CuL}]}{[\mathrm{Cu}^{2+}{}_f]} = \frac{[\mathrm{L}]K}{1 + K[\mathrm{Cu}^{2+}{}_f]}. \tag{6}$$

We used the program RS-1 (BBN Software) to solve Eq. 6 for K and [L] by nonlinear regression analysis with $Cu^{2+}{}_f$ as the independent variable and $\Sigma K_i L_i/[Cu^{2+}{}_f]$ as the dependent variable. In reality, because weaker ligands are present in the media (such as carbonate and weak, naturally occurring ligands), a more correct form of the equation would be

$$\frac{\Sigma[\mathrm{CuL}_i]}{[\mathrm{Cu}^{2+}{}_f]} = \Sigma K_i \mathrm{L}_{i(i>1)} + \frac{[\mathrm{L}_1]K_1}{1 + K_1[\mathrm{Cu}^{2+}{}_f]}. \tag{7}$$

$\Sigma K_i L_{i(i>1)}$ is the side-reaction coefficient for the weaker ligands, and K_1 and L_1 represent K and L in Eq. 6. In practice, this term could be neglected when one determines K and [L] except in the samples containing 5×10^{-6} M NTA, when we used a value of 1,000 for $\Sigma K_i L_{i(i>1)}$.

Figure 2 shows plots of $\Sigma[CuL_i]/[Cu^{2+}{}_f]$ vs. $[Cu^{2+}{}_f]$ for four *Synechococcus* cultures. The solid line represents the nonlinear least-squares regression fit to the data using Eq. 7. In all cases the single ligand model provides a good fit to the data, suggesting that a single ligand or binding site probably predominates in these cultures. The data were obtained for three different bzac concentrations; note that results at all three concentrations were consistent. The titration data should be independent of bzac concentration if the system is at equilibrium, so this is an important

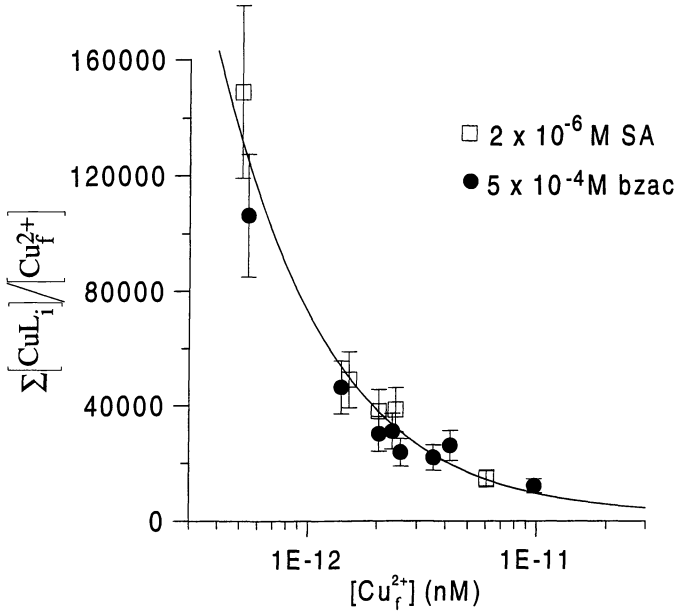

Fig. 3. Comparison of titration data obtained with SA and bzac. Graph shows a filtered DC-2 culture plotted as a reciprocal Langmuir isotherm. This sample contained 5×10^{-6} M NTA.

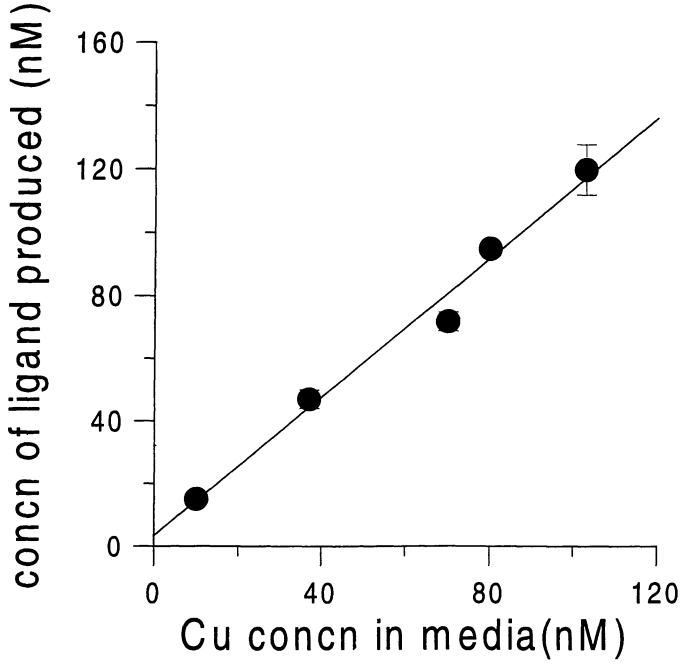

Fig. 4. The concentration of strong ligand produced vs. dissolved Cu concentration in the filtrate. Ligand concentrations were determined from the titration data (*see Fig. 2 and Table 3*).

observation. Likewise, the results in Fig. 3 show that titrations of the same culture filtrate with 2×10^{-6} M SA and 5×10^{-4} M bzac yield the same results within experimental error. Results of these and other titrations are summarized in Table 3.

Estimates of the conditional stability constants range from 2.2×10^{13} to 2.0×10^{12}—an order of magnitude. Does this mean that there are multiple ligands in the system, with weaker ligands predominating at higher Cu concentrations? Multiple ligands are certainly possible, but there may be a systematic artifact associated with the derivation of K that could account for the differences that arise. The ideal Cu titration data set for deriving conditional stability constants would begin at [Cu] $\ll$ [L]. With such a data set, linearization methods, such as the Scatchard and van den Berg/Ruzic plots, give very accurate measurements of K from the low-Cu end of the titration. In our experiments, by contrast, the ligand is almost completely saturated with Cu, and it becomes harder to distinguish a strong, completely saturated ligand from a much weaker ligand that is less saturated. With a ligand exchange method, however, much of the Cu initially present can be coordinated by the competing ligand, with the result that $\Sigma[CuL]/[Cu^{2+}{}_f]$ can be determined at residual Cu values lower than the Cu initially present in the sample. In cultures containing the lowest concentrations of ligand, many data points were obtained at [Cu]<[L]. However, at the higher ligand concentrations, only a much smaller relative fraction of the ambient Cu could be complexed by bzac, and the necessary data points below the ambient metal concentration could not be obtained. Given this caveat, the results are consistent with the previous estimate of log K = 12.8 for the ligand produced in non-Cu-spiked DC-2 culture (Moffett et al. 1990).

Table 3. Total Cu concentration, ligand concentrations, and conditional stability constants for filtrates of *Synechococcus* cultures (clone DC-2). All concentrations in nM.

[Cu]	[L] (Eq. 6)	[L] (Scatchard)	K
A. No NTA in growth media			
10±2	15±2	15	$(2.2 \pm 0.8) \times 10^{13}$
40±3	47±3	45	$(1.2 \pm 0.4) \times 10^{13}$
70±4	72±3	65	$(3.9 \pm 1) \times 10^{12}$
81±4	95±2	102	$(4.3 \pm 0.2) \times 10^{12}$
103±4	120±8	110	$(2.0 \pm 0.6) \times 10^{12}$
B. 5×10^{-6}M NTA			
112±5	110±6		1.05×10^{12}
139±6	85±20		2.2×10^{13}
166±6	140±14		1.6×10^{12}

Determining the ligand concentration is more straightforward because the complexes are very strong, and there is nothing comparable in the media. Estimates of ligand concentrations obtained by nonlinear regression and from the initial, linear portion of the Scatchard plots are shown in Table 3 and are in good agreement.

Figure 4 shows a plot of ligand concentration (from nonlinear regression) vs. dissolved Cu in the filtrate for the DC-2 cultures studied in the absence of NTA (including the four titrations shown in Fig. 2). The concentration of strong ligand increased with increasing Cu concentration, and there was nearly a 1 : 1 relationship be-

tween the amount of ligand produced and the dissolved Cu concentration, with a slight excess of ligand over dissolved copper.

Because of the sensitivity of *Synechococcus* to Cu toxicity, only small increments of Cu could be gradually added to the samples. Despite this sensitivity, cultures occasionally died. To overcome this problem, we conducted further experiments with 5×10^{-6} M NTA in the media. This amount produced a side-reaction coefficient of 1,000, which was not large enough to lower free Cu to nontoxic levels, but kept it at levels 10–20× lower than the unbuffered media. NTA was added for several experiments with DC-2 (including the data shown in Fig. 3). Results were similar to those in the unbuffered samples, except that the organisms did not make as much ligand relative to the total Cu. This result was not surprising because in the presence of NTA, less ligand would be required to lower Cu to nontoxic values. In addition, we surveyed the three other *Synechococcus* clones to be certain that ligand production was not an anomalous characteristic of DC-2. All clones studied produced strong ligand in response to Cu stress, suggesting that this response is a general feature of *Synechococcus* in diverse marine environments. Figure 5 shows titration data for the remaining three clones in experiments performed with 2 μM SA. Estimates of K for A3844 and A2357 determined by nonlinear regression were comparable to DC-2 culture data. The estimate of K for A2346 was a much higher, thermodynamically meaningless value, reflecting the sensitivity of the fit to the single data point at the beginning of the titration.

The production of chelators by eucaryotic algae is currently being investigated. None of the species studied produced any detectable strong chelator in the absence of added Cu. However, in the presence of Cu, several species, most notably *Amphidinium carterae* and *Skeletonema costatum,* produced a weaker chelator (log $K \sim$ 11) at concentrations comparable to the ambient Cu concentration; however, this could have been a much stronger chelator produced at concentrations well below the ambient Cu concentration. Work in progress on eucaryotic chelators will be described elsewhere.

Discussion

Synechococcus clearly benefits from the presence of the strong chelator as shown in Fig. 6, which plots growth rates in culture vs. free Cu from Brand et al. (1986) with the range of free cupric ion determined in the media from our experiments superimposed. The range of free Cu in the media if no chelator were produced is clearly high enough to stop all growth of the organism (Fig. 6). How-

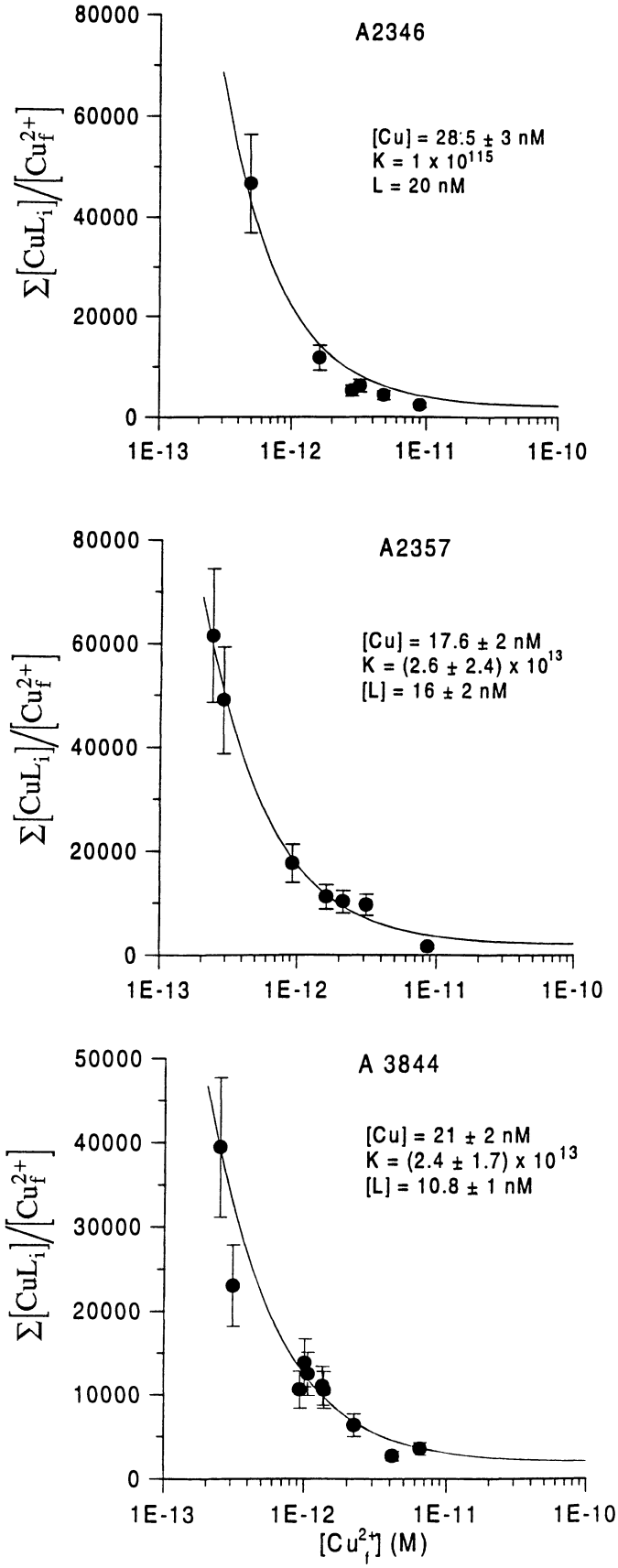

Fig. 5. Reciprocal Langmuir isotherm plots for Cu stressed cultures of *Synechococcus* clones A2346, A2357, and A3844. All data were obtained with 2×10^{-6} M SA. All cultures contained 5×10^{-6} M NTA; note different scales.

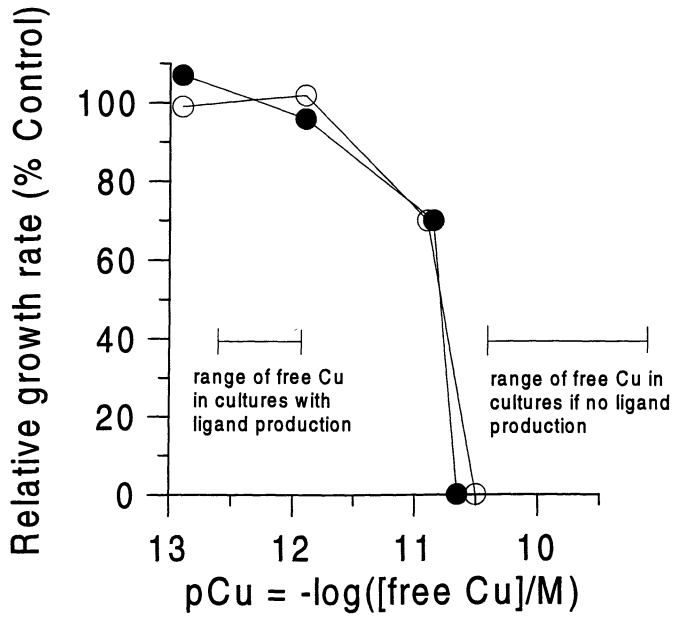

Fig. 6. Growth rate of *Synechococcus* sp. at different concentrations of $Cu^{2+}{}_f$ (*from* Brand et al. 1986). Symbols depict results of replicate experiments. Superimposed are the ranges of $[Cu^{2+}{}_f]$ in the cultures and the predicted ranges had no ligand been produced.

ever, by producing the chelator, the organism would be able to keep free cupric ion levels at concentrations that do not affect growth rates. Note that the growth rate measurements shown in Fig. 6 were carried out in strongly metal ion-buffered media that the organisms were unable to modify (Brand et al. 1986).

Synechococcus sp. is the only species we have identified that produces compounds with the requisite high conditional stability constants (log $K > 13$) to be a plausible source of L1. None of the eucaryotes produced this material, although they seemed to produce weaker chelators in the presence of added Cu. Moffett et al. (1990) studied three eucaryotic species, *Hymenomonas carterae, Thalassiosira pseudonana,* and *Micromonas pusilla*; none produced strong chelators in non-Cu-spiked cultures, a finding consistent with the present study. *Synechococcus* was the only procaryote studied, but a variety of procaryotes may produce these compounds.

Our results are in general agreement with an extensive investigation of freshwater algal exudate production conducted by McKnight and Morel (1979). They did not find any strong chelator production in cultures of eucaryotes, but detected a strong chelator ($K > 10^{10}$) in a culture of *Synechococcus leopoliensis.*

The close relationship between the ligand and Cu concentrations in Fig. 4 could result from a feedback mechanism whereby ligand production is induced when free Cu levels exceed some threshold value and is discontinued when free Cu drops below this value. An alternative mechanism could involve complexation of Cu with intracellular or surface-bound chelators. If the resultant Cu complexes "leak" out or are excreted, eventually all of the dissolved Cu will become complexed, and the result would be indistinguishable from a mechanism in which a free ligand is exuded. This mechanism could explain the accumulation of Cu on or within the cells, which we frequently observed in the addition experiments (the data in Fig. 2 show dissolved Cu in the filtrate, but this amount was always 20–50% less than the Cu initially added). Radiotracer studies have shown that Cu-resistant freshwater cyanobacteria can export intracellular Cu into the media (Verma and Singh 1991). This mechanism could explain our data if the Cu is exported as complexes; however, such a mechanism would not account for a significant excess of L1 over total Cu—a typical situation in surface ocean waters.

Table 4. Some recent stability constant estimates for class 1 ligands in oceanic waters.

log$K$	Location	Reference
13.2±0.2	S. and central Sargasso Sea	Moffett 1995
13.0±0.6	NE Pacific	Coale and Bruland 1990
13.2	SE continental shelf	Sunda and Huntsman 1991
13.2±0.2	S. Sargasso Sea	Moffett et al. 1990
12.6±0.5	Indian Ocean	Donat and van den Berg 1992

The ligand produced in these experiments is probably the same compound that we detected in the earlier study with no added Cu (Moffett et al. 1990) because the conditional stability constants are so similar. The values (Table 3) are in close agreement with estimates of the conditional stability constants for L1 measured by various groups in upper oceanic water columns (Table 4). Furthermore, the estimates from Moffett (1995) were measured by the same method in the same laboratory as the results reported here. However, similarity in conditional stability constants alone does not constitute proof that L1 is produced by *Synechococcus.*

The production of extracellular Cu chelators by two freshwater cyanobacteria in response to Cu stress has been reported (Jardim and Pearson 1984). However, the chelators were weak (log$K$ = 6–7 compared to 13 in this study), so the chelators probably do not represent a detoxification strategy in the water column because millimolar levels of this chelator would need to be produced to have a significant effect on Cu speciation.

What is the nature of the chelators produced by *Synechococcus*? Various metal-binding compounds have been detected in cultures of marine and freshwater cyanobacteria. These compounds include H_2S (Dunstan et al. unpubl.), a catechol siderophore (Wilhelm and Trick 1994), and metallothioneins (Olafson et al. 1980). However, we found no evidence that any of these compounds are the strong ligand reported here. Cu is not displaced from the strong ligand by Fe(III) even at a 100-fold excess, so the ligand is unlikely to be a siderophore. Similarly, Cd was unable to displace Cu from the ligand, even at a

100-fold excess. Eucaryotic marine phytoplankton have been shown to produce phytochelatins, which are strong Cu chelators (Ahner and Morel 1995). However, no phytochelatins have been detected in cultures of *Synechococcus* (B. Ahner pers. comm.).

If cyanobacteria do modify their aquatic chemical environment in this way, it would have important implications for the role of metals in marine ecosystems. Such modification suggests that the hypothesis originally proposed by Barber and Ryther (1969) that seawater must be "conditioned" by organisms to provide conditions optimal for growth is applicable. Conditioning may be particularly relevant for a class of organisms—the cyanobacteria—whose importance in marine ecosystems had not been recognized when Barber and Ryther proposed their hypothesis.

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